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Evaluation of Androgen Receptor Function in Prostate Cancer Prognosis and Therapeutic Stratification

PRINCIPAL INVESTIGATOR:
Albert Dobi, PhD

CONTRACTING ORGANIZATION : Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc.
Bethesda, MD 20817

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14. ABSTRACT While prostate cancers initially respond to androgen ablation therapy, tumors often become treatment resistant as tumor cells develop mechanisms to evade the treatment. Dysfunction of the androgen receptor frequently observed in castration resistant stages of prostate cancer. We reasoned that early knowledge of androgen receptor dysfunction can predict the course of prostate cancer progression. We proposed an approach for monitoring potential dysfunctions of the androgen receptor by measuring the expression of a panel of genes directly regulated by androgen receptor. We examined human prostate cancer tissues (surgery specimens) at early stages of the disease and matched with longitudinal follow up data. We have completed the evaluation of 140 patients by monitoring mRNA expression levels of <i>ERG</i> , <i>PSA</i> , <i>PMEPA1</i> , <i>ODC1</i> , <i>AMT1</i> and <i>GAPDH</i> levels. Also, we have completed the evaluation of 80 whole-mounted sections of radical prostatectomy specimens by immunohistochemistry assessing AR, ERG, NKX3.1 and PSA proteins. This study addresses the association of AR function defects (decreased or attenuated expression of AR regulated genes) with unfavorable clinical features, as well as, if expression levels of the androgen regulated gene panel is indicative of biochemical recurrence- and metastasis-free survival. Major findings emerging from our PCRP/CDMRP award revealed that monitoring the levels of AR regulated proteins may support disease progression-associated clinical-pathologic features. In contrast, assessment of AR regulated genes at mRNA levels show promising performance in supporting the prediction of biochemical recurrence –free survival in low grade tumors. In summary, the study results indicate the utility of monitoring the defects of AR function in prostate cancer.					
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1. INTRODUCTION:

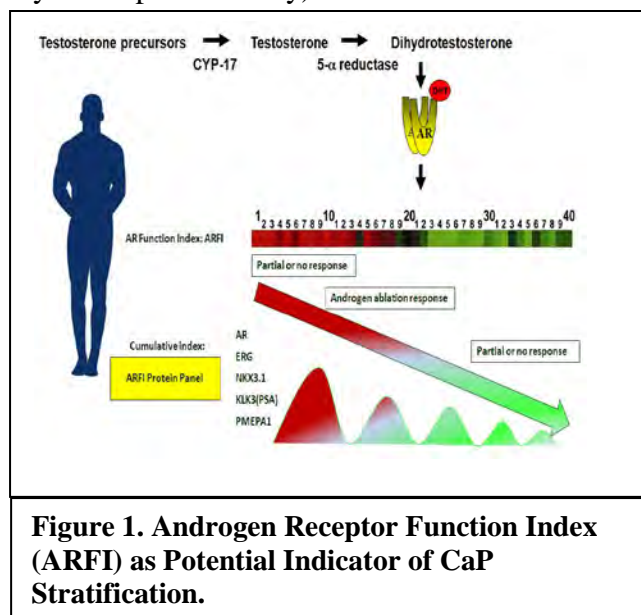
This PCRP/CDMRP/DOD Idea Development Award supported project was initiated in 2011. The original award was for three years and was granted with a one year no-cost extension. This final report encompasses the entire project period from 2011 to 2015 and delineates the potential clinical application of findings resulted from the accomplishments of the award supported research.

The proposal's idea originated from the clinical observation that prostate cancer is initially driven by the male hormone androgen acting through the androgen receptor. However, in some patients androgen receptor becomes dysfunctional at late stages of tumorigenesis. These patients are less likely to show sustained response to androgen ablation therapy and more likely to die from incurable metastatic prostate cancer. Thus, early knowledge of the androgen receptor dysfunctions, that would make prostate tumors refractory to routine androgen ablation therapy, should help in patient stratification for new emerging therapeutic strategies. Indeed, while prostate cancers initially respond to androgen ablation therapy, tumors often become treatment resistant as tumor cells develop mechanisms to evade the treatment. In this castration resistant (CRPC) stage of prostate cancer the dysfunction of the androgen receptor (AR) has been recognized (Schmidt and Tindall Curr Drug Targets 2013; Dobi, Sreenath et al., Book chapter 2013; Yuan et al., Oncogene 2014; Crawford et al., J Urol 2015).

The scope of this proposal is to predict the course of prostate cancer progression by monitoring a panel of AR regulated genes. To monitor the functional status of androgen receptor in prostate tumors a panel of six androgen inducible genes were selected (Xu et al., J Urol 2000; Xu et al., Genomics 2000; Segawa et al., Oncogene 2002). This panel includes tissue (*KLK3*(PSA)), AR stability regulator (*PMEPA1*), transcription factor (*NKX3.1*), polyamine biosynthesis (*ODC1*, *AMD1*) and oncogene (*ERG*) protein coding genes. Selected genes are either direct targets of AR or are tightly regulated by AR and have demonstrated prostate associated expression encompassing major biological functions regulated by AR in the human prostate. Expression levels of the selected genes were determined in radical prostatectomy specimens. CPDR Biospecimen Bank linked to comprehensive clinico-pathologic patient data base and longitudinal follow-up were used for evaluation of quantitative gene expression values in RP specimens for 1) positive or negative correlation with disease progression as defined by Gleason grade, pathological stage and biochemical recurrence; 2) feasibility of gene expression measurement in clinical assays (specificity, sensitivity and reproducibility).

We reasoned that AR function in prostate tumor cells can be precisely defined by measuring the expression of AR regulated gene panel (ARP), including (*KLK3* (PSA), *PMEPA1*, *TMPRSS2-ERG* (*ERG*), *NKX3.1*, *AMD1* and *ODC1*) towards defining AR function by the Androgen Receptor Function Index (ARFI) (Figure 1).

In our approach we leveraged the mechanistic understanding of direct AR regulated genes. Thus, our approach is distinct from the empirically design OncotypeDX Prostate mRNA-based AR-regulated gene set that has been recently introduced to clinical practice by Genomic Health Inc.



A second distinctive feature and major strength of promising data emerging from our PCRP/CDMRP/DOD award is that monitoring AR function at the protein expression levels of panel of androgen regulated genes may provide an IHC-based more easily adaptable and cost effective approach in routine pathology settings. In our approach we have addressed the association of AR function defects with unfavorable clinical features, if the ARFI is indicative of BCR and metastasis-free survival. Leveraging the high representation of African American men in the CPDR data and tissue bank we evaluated the performance of monitoring AR-regulated genes in both Caucasian American and African American men in response to the emerging need for biomarkers that performs equally well in ethnic groups within the United States. We have completed a collaborative evaluation of castration resistant prostate cancers (CRPC). In support of the central hypothesis of this proposal, the result revealed a thus far unrecognized type of CRPCs with dispensed AR function. In summary, the observed readouts of AR dysfunction may provide a promising tool for improved prognostic accuracy and patient stratification at early stages of prostate cancer.

The objective of this proposal is to predict the course of prostate cancer progression by monitoring a panel of AR regulated genes in stratifying patients for treatment modalities. We have reported the association of decreased expression of androgen regulated genes with features of prostate cancer progression (Sterbis, Gao et al. 2008; Dobi, Furusato et al. 2010; Sharad Epigenetics 2014; Gsponer et al, 2014). Other reports have also noted a signature of attenuated AR function in late stage, especially in metastatic prostate cancer in human specimens (Varambally, Yu et al. 2005), (Hermans, van Marion et al. 2006), (Tomlins, Mehra et al. 2007) (Mendiratta, Mostaghel et al. 2009). Assessment of AR function by measuring the expression of androgen regulated genes as part of a gene panel has been recently shown to improve the prediction of the presence or absence of adverse pathology at the time of diagnosis (Klein et al., Eur Urol 2014; Cullen et al., Eur Urol 2014).

To develop readouts for AR function in CaP cells, this PCRP/CDMRP Idea Development grant award focused on the quantitative measurements of AR regulated genes in carefully isolated benign and tumor cells and in prostate tumor tissues. The findings reported here summarize the accomplishments of the proposal towards the evaluation of androgen receptor function towards the following specific aims:

Aim #1: To establish the Androgen Receptor Function Index (ARFI) by assessing the AR regulated gene panel as a quantitative measure of in vivo AR functional status in prostate cancer at the time of radical prostatectomy.

Aim #2: To define the utility of AR-regulated proteins by the AR Function Index, ARFI.

2. KEY WORDS:

Prostate cancer, androgen receptor, androgen receptor regulated genes, prostate cancer progression PSA, NKX3.1, PMEPA1, ERG, ODC1, AMD1

3. ACCOMPLISHMENTS:

a) Specific objectives and major activities

Towards Aim#1, Task 1: to establish the Androgen Receptor Function Index (ARFI) by assessing the AR regulated gene panel (ARP) as a quantitative measure of in vivo AR functional status in prostate cancer at the time of radical prostatectomy.

Step 1 plan (Months 1-6):

Institutional Review Board regulatory review and approval processes will occur at two separate institutions: the Walter Reed Army Medical Center (WRAMC) and the Uniformed Services University of the Health Sciences (USUHS). While IRB approval is still ongoing, probes and primers will be designed following the same principles we have been using for TaqMan and will be tested for specificity and sensitivity by using copy number defined dilutions of cDNA clones of ARP.

Completed IRB approval in Month 10 and the Task completed by Month 12 (100%).

Step 2 plan (Months 6-22):

RNA samples for QRT-PCR will be obtained from radical prostatectomy specimens of 110 CaP patients following our established strategy. Total RNA from laser-captured microdissected (LCM) normal and cancer cells from either formalin fixed paraffin embedded (FFPE) or OCT embedded and H&E stained frozen prostate sections of radical prostatectomy specimens (5-10,000 epithelial cells per sample) will be acquired from the CPDR Biospecimen Bank. Total RNA will be quantified by using RiboGreen fluorometric method. The total RNA isolated from the paired tumor and normal LCM epithelium specimens will be converted to cDNA. The expression levels of ARP genes (*PSA/KLK3*, *PMEPA1*, *NKX3.1*, *ODC1*, *AMD1* and *ERG/TMPRSS2-ERG*) will be determined in the matched tumor and normal prostate tissue-derived cDNA samples by real time QRT-PCR (TaqMan).

Completed the mRNA expression analyses of 140 tumor samples (77 cases by QRT-PCR, 63 cases by NanoString) by Month 40 (100%): Total RNA from laser-captured microdissected (LCM) normal and cancer cells from both formalin fixed paraffin embedded (FFPE) and OCT embedded and H&E stained frozen prostate sections of radical prostatectomy specimens were acquired from the CPDR Biospecimen Bank. Total RNA were quantified by using RiboGreen fluorometric method. The total RNA isolated from the paired tumor and normal LCM epithelium specimens were converted to cDNA. The expression levels of AR-regulated genes (*PSA/KLK3*, *PMEPA1*, *NKX3.1*, *ODC1*, *AMD1* and *ERG/TMPRSS2-ERG*) were determined in the matched tumor and normal prostate tissue-derived cDNA samples from frozen tissues by real time QRT-PCR (TaqMan). In a new approach the study design was focused on low grade disease (Gleason 3+3 or 3+4; no positive margins, no extracapsular extension, and no seminal vesicle invasion) with (N= 21) or without (N=42) recurrence (BCR or Mets). NanoString analysis was performed on 63 macrodissected tumor cDNA samples. The advantage of the NanoString platform is the reliable quantitative data by mRNA copy number on FFPE-derived mRNA in multi-plex analyses. The expression levels of AR-regulated genes (*PSA/KLK3*, *PMEPA1*, *ODC1*, *AMD1* and *ERG*) were determined in 63 macrodissected FFPE-derived tumor samples.

Step 3 plan (Months 23-40):

AR panel expression data will be analyzed by informatic and statistical methods for positive or negative correlation with aggressiveness of prostate cancer, as defined by Gleason grade, pathological stage, biochemical recurrence and for feasibility of gene expression measurement in a clinical assay (specificity, sensitivity and reproducibility). Cumulative index will be used for quantitative definition of AR function (ARF index) towards determine the stratification power of AR gene panel at the time of radical prostatectomy. This index will be incorporated into nomograms modeling time-to-event data, including prediction of disease progression, combined with established clinical and pathological characteristics that predict this study endpoint.

Completed the QRT-PCR evaluation of ARP mRNA expression data showing correlation between the expression of AR regulated genes. However, the analysis did not show correlation of cumulative mRNA levels as defined by QRT-PCR with disease progression. Taking a new, more quantitative approach using NanoString platform the analyses of ARP mRNA in FFPE-derived tumor samples (N=63) revealed 74% specificity and 76% sensitivity for predicting BCR-free survival among patients with low-grade index tumors: Gene AR panel expression data were analyzed by informatic and statistical methods for positive or negative correlation with aggressiveness of prostate cancer, as defined by Gleason grade, pathological stage, biochemical recurrence and for feasibility of gene expression measurement in a clinical assay including specificity and sensitivity. Cumulative index were used for quantitative definition of AR function towards determine the stratification power of AR gene panel at the time of radical prostatectomy. Due to the low number of events incorporation of ARFI into nomograms (modeling time-to-event) was not feasible. However we were able to complete the assessment of ARP specificity and sensitivity for predicting disease progression in low grade disease.

Towards Aim#2, Task 2: to define the utility of AR-regulated proteins by the AR Function Index, ARFI.

Step 1 plan (Months 12-30):

IHC will be set up and optimized with antibodies against ARP gene products. Whole-mounted sections of RP specimens with prostate cancer will be assayed in a cohort of over 110 patients by immunohistochemistry. The staining intensities will be determined according to percent of cells positive. The intensity will be scored and a combination of measurements will be calculated by multiplying the percent of positive cells with the degree of intensity, which will result in an IHC intensity score. The sum of staining intensity scores will be expressed as the cumulative IHC staining index of AR regulated gene panel.

Completed the evaluation of index tumors of 80 whole-mounted radical prostatectomy specimen indicating 74% specificity and 54% sensitivity in predicting biochemical recurrence and/or metastasis among Caucasian American men. The observed 74% specificity in detecting AR dysfunction may support the early identification of patients with higher risk for progression among CA patients. Attenuated expression of PSA and NKX3.1 was consistently observed in index tumors of both AA and CA men with Gleason score 8-10 carcinoma. Although the overall pattern of AR regulated genes were similar except ERG, that was absent in majority of index tumors of AA men the panel showed only 52% specificity and 47% sensitivity for predicting BCR/Metastasis in AA men: IHC was optimized with antibodies against AR regulated gene protein products (PSA, NKX3.1, ERG and AR). Whole-mounted sections of RP specimens with prostate cancer were assayed in a cohort of 80 patients by immunohistochemistry. The staining intensities were determined according to percent of cells positive. The intensity was scored and combination of measurements was calculated by multiplying the percent of positive cells with the degree of intensity, which resulted in an IHC intensity score. The sum of staining intensity scores were expressed in a four color scale as the cumulative IHC staining index of AR regulated gene panel.

Step 2 plan (Months 12-40):

To establish concordance between the expression of ARP mRNA and proteins branched-chain DNA (b-DNA) signal amplification method will be used. Adjacent 4 µm-thick section will be selected from each of the FFPE whole-mount prostate samples.

The b-DNA approach in this step was replaced with microarray analyses comparing ARFI mRNA to immunostaining of ARP proteins in adjacent sections of whole mounted prostates.

Step 3 plan (Months 31-44):

Incorporate the AR gene panel cumulative indexes into predictive nomograms for prostate cancer progression defining predictive power and utility for patient stratification. Protein ARP protein panel will be assessed in formalin fixed paraffin embedded (FFPE) tissues by evaluating 110 whole-mounted radical prostatectomy specimens. From the IHC staining of products of the ARP will be summarized in a cumulative index for patient stratification. IHC scores and a cumulative pathology scores will be established for the tumor foci in the sections. Cumulative IHC score will be evaluated alone and by combining with nomograms modeling time-to-event data incorporating the biochemical recurrence within eight years of follow up. Post-operative predictive value of existing, validated nomograms will be assessed by using the patient cohort. The IHC-derived ARP values, as a single cumulative index, will be incorporated into validated nomograms (Kattan) modeling time-to-event data, including prediction of CaP progression, combined with established clinical and pathological characteristics that predict this study endpoint. The concordance index, will be used to assess the improvement in model fit upon inclusion of AR function index.

Completed the development of a numeric IHC scoring system from the assessment of 80 whole-mounted radical prostatectomy specimens and the evaluation of sensitivity and specificity of ARP IHC in predicting BCR/distant metastasis. From the IHC staining of products of the ARP were expressed in a four color scale representing the cumulative index. IHC scores and disease progression (BCR/metastasis) were correlated to ARP. Due to the low number of events nomograms, modeling time-to-event, was not feasible. However, the performance characteristics of ARP IHC was calculated resulting in 74% specificity and 54% sensitivity in predicting BCR/distant metastasis in support of unfavorable clinical-pathologic features. This performance was observed only among CA patients.

b. Significant results and key outcomes:

By the end of the first reporting period Institutional Review Board approval was obtained from the Walter Reed National Military Medical Center (WRNMMC) and from the Uniformed Services University of the Health Sciences (USUHS). Primers and probes were prepared and QC-ed for qRT-PCR assays for assessing the expression of *PSA/KLK3*, *PMEPA1*, *NKX3.1*, *ODC1*, *AMD1*, *AR* and *ERG (TMPRSS2-ERG)* genes. The quality control of primers and probes for the detection of endogenously expressed AR panel genes has been completed, target sequences were confirmed by DNA sequencing of PCR products and sensitivity and specificity was confirmed.

To monitor the functional status of androgen receptor in prostate tumors a panel of six androgen inducible genes were selected. This panel includes tissue (*KLK3(PSA)*), AR stability regulator (*PMEPA1*), transcription factor (*NKX3.1*), polyamine biosynthesis (*ODC1*, *AMD1*) and oncogene (*TMPRSS2-ERG*) protein coding genes. Selected genes are either direct targets of AR or are tightly regulated by AR and have

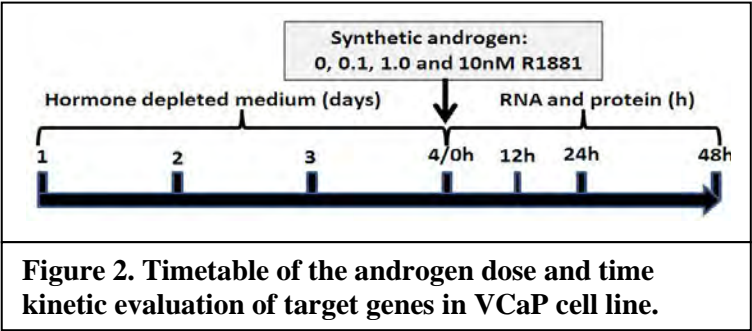


Figure 2. Timetable of the androgen dose and time kinetic evaluation of target genes in VCaP cell line.

demonstrated prostate associated expression encompassing major biological functions regulated by AR in the human prostate. The primers and probes have been obtained, and tested by using cDNA from VCaP prostate cancer cell line endogenously expressing all target genes, *PMEPA1* (Locus ID: GXL_128240), *TMPRSS2-ERG* fusion A (Locus ID: GXL_39091), *KLK3*(PSA) (LocusID: GXL_32002), *NKX3.1* (LocusID: GXL_29827), *AMD1* (Locus ID: GLX_261249, and *ODC1* (Locus ID: GXL_75806). PCR amplicons were isolated and target regions were confirmed by DNA sequencing. To assess the performance of primers and probes assessing androgen dose and time kinetic response of target genes, VCaP cells were grown in androgen depleted conditions for four days. To induce androgen regulated genes, synthetic androgen (R1881) was added to the cell cultures at 0, 0.1, 1.0 and 10nM concentrations and cells were harvested at 0, 12, 24 and 48h time points (Figure 2.).

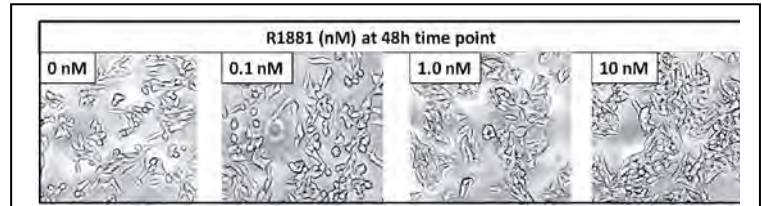


Figure 3. VCaP cell morphology in response to increasing doses of R1881 treatment at 48h (20X magnification).

To monitor changes in cell morphology in response to R1881 induction, cells were assessed at 0, 12, 24 and 48h time points. The observed cell morphology was consistent with the expected time and dose kinetic response of VCaP cells to R1881 treatment (Figure 3.).

Evaluating protein products of three of the target genes (*PSA*, *NKX3.1* and *ERG*) that was previously shown robust response to androgen induction in hormone responsive prostate cancer cell culture models (Xu et al., 2001; Segawa et al., 2002; Tomlins et al., 2005), VCaP cells were harvested and cell lysates were prepared at 0, 12, 24 and 48h time points in the 0, 0.1, 1.0 and 10 nM R1881 treatment groups. Expression of *PSA*, *NKX3.1* and *ERG* proteins were assessed by immunoblot assays. As expected, the experiment revealed androgen hormone dose dependent expression of the assayed proteins (Figure 4.).

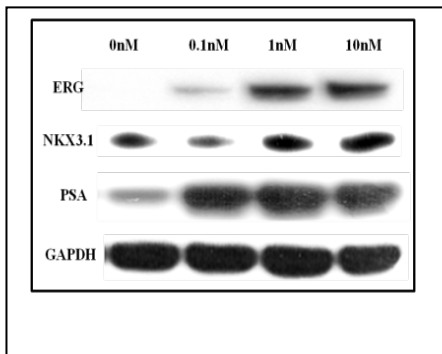


Figure 4. Expression of ERG, NKX3.1 and PSA protein in response to increasing doses of R1881 at 48h time point in VCaP cells. GAPDH was used as the loading control in the immunoblot assay.

For the evaluation of ARP target gene expressions RNA was isolated from VCaP cells at 0, 12, 24 and 48h in the 0, 0.1, 1.0 and 10 nM R1881 treatment groups. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in triplicates and one control reaction without RT were performed for each RNA sample by using primer and probe sets for *KLK3*(PSA), *TMPRSS2-ERG*(fusion junction “A” (Tomlins et al., 2005)), *NKX3.1*, *PMEPA1*, *ODC1* and *AMD1* genes and expression values were expressed relative to *GAPDH*. Expression results were calculated from the average CT (threshold cycle) values of triplicates (Figure 5.). Specific primers and probes were designed for the qRT-PCR analyses of *PSA/KLK3*, *PMEPA1*, *NKX3.1*, *ODC1*, *AMD1* and *TMPRSS2-ERG* genes. Quality control of primer and probe sets has been completed. Kinetic and androgen dose response of endogenously expressed levels of ARFI genes in VCaP cells indicate the sensitivity of detection. Sequence analyses of target gene amplicons confirmed the specificity of detecting ARFI genes.

In Year#2 of the award RNA samples for qRT-PCR were obtained from radical prostatectomy specimens from 77 patients enrolled in the Center for Prostate Disease Research (CPDR) program between 1996 and 2010. Clinicopathologic data were obtained from the CPDR database. Optimum cutting temperature (OCT) embedded RP tissues specimens from 42 Caucasian American (CA) and 35 African American (AA) men due to reported differences in incidence and mortality. None of the patients had received androgen deprivation therapy. Biochemical recurrence (BCR) was defined as two consecutive post-operative PSA values greater than 0.2 ng/mL measured at ≥ 8 weeks post-operatively. Total RNA from laser-captured microdissected (LCM) normal and matching cancer cells from OCT embedded and H&E stained frozen prostate sections of radical prostatectomy specimens were prepared. Total RNA were isolated from paired tumor and normal LCM epithelium specimens. Expression levels of AR panel genes (*PSA/CLK3*, *PMEPA1*, *ERG*(*TMPRSS2-ERG*) and *GAPDH*) were determined by real time QRT-PCR (TaqMan). Clinico-pathologic data were obtained from the CPDR database. **The analysis showed positive correlation between the expressions of ARP genes (*ERG* vs. *PMEPA1* and *ERG* vs. *PSA*) in tumors of AA patients that has not observed within the CA group. We have further extended the focused evaluation of *PMEPA1* gene due to its emerging role in regulating AR activity (Sharad et al., Epigenetics 2014).**

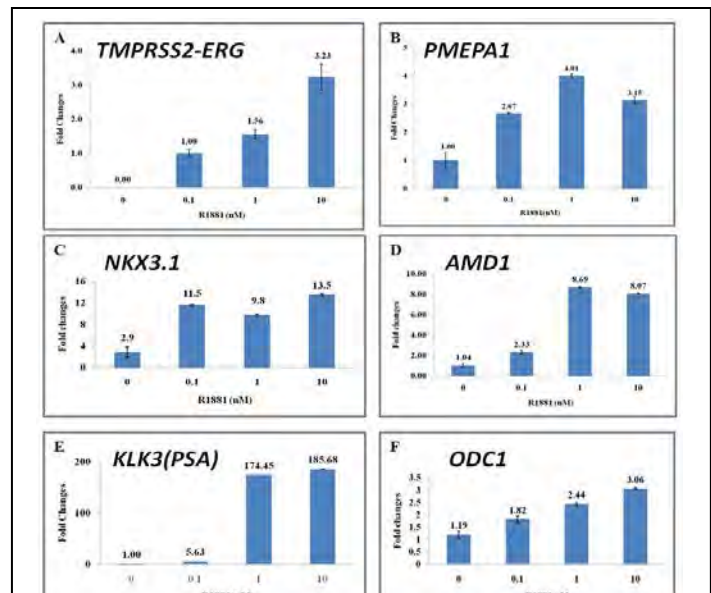


Figure 5. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of *CLK3(PSA)*, *TMPRSS2-ERG*(*ERG*), *NKX3.1*, *PMEPA1*, *ODC1* and *AMD1* genes in VCaP cells demonstrate the activation of ARFI genes in response to increasing doses of R1881 at 48h time point. Experiments were performed in triplicates and expression levels relative to *GAPDH* are shown as fold changes.

To assess the concordance between the expression of ARP mRNA and proteins we have completed the direct comparison of ARP IHC results from index tumors of 40 prostate cancer cases to mRNA expression data obtained by Affymetrix microarrays. The result showed 90% concordance between detecting *ERG* protein or *ERG* mRNA validating the identical origin of tumor samples assessed by IHC and by gene expression experiments. However, the overall concordance between the cumulative indexes of AR panel proteins and the expression of AR panel genes were only 50% likely due to notable differences between the protein and mRNA levels of the *NKX3.1* and *PSA (CLK3)* genes and to challenges in matching frozen ex-vivo biopsies used in mRNA expression to the protein assessments by IHC of index tumors in whole mounted RP specimens. (Figure 6 and 7).

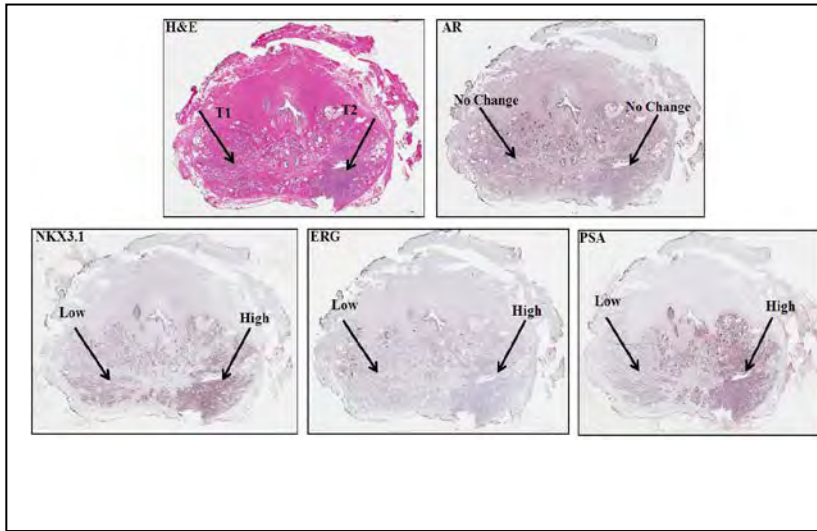


Figure 6. Evaluation of whole-mount prostate sections for the expression of ARP genes by immunohistochemistry. On the upper left panel two tumor foci T1(index) and T2 are shown by H&E. The upper right panel shows equal AR immunostaining in both tumor foci (no change). Lower panels show reduced immunoreactivity of NKX3.1, ERG and PSA in the left tumor focus (Low), in contrast to the tumor focus shown at the right side (High) of the whole-mount prostate section.

Staining intensities have been determined and intensity was scored and a combination of measurements was calculated. **Assessment of ARP genes by IHC showed remarkable accuracy in identifying biochemical recurrence (BCR) and metastasis in tumors with poorly differentiated (PD) morphology (14 out of 15 BCR/metastasis). In contrast, detection of RNA levels of ARP genes performs better in predicting favorable outcome in tumors with well differentiated (WD) morphology, precisely identifying 13 cases with BCR-free survival (no-BCR) and no metastasis out of 14 cases classified as intact AR (Figure 7).**

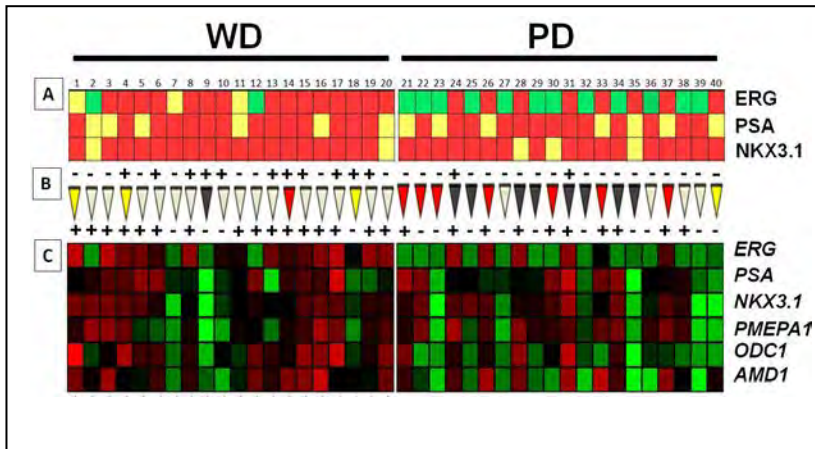


Figure 7. (A) Attenuated expression are frequently (85%) found in tumors with poorly-differentiated (PD, patient 21-40) morphology as indicated by the IHC heatmap of index tumors in 40 prostate cancer cases (Red: IHC positive (95%-100% & 2-3+); Yellow: intermediate, 1-2+); Green: negative). (B) BRC (red and black triangles), no recurrence (white triangles) or no follow up data (yellow triangles) symbols are aligned with the cumulative ARP index on top (“+” marks intact AR signaling; “-“ indicates that at least one of the AR readout proteins in the panel is absent or attenuated in IHC). (C) Gene expression (GeneChip) data of the corresponding index tumors indicates elevated (red) decreased (green) or unchanged (black) expression values of the AR regulated genes. According to the cumulative index definition AR signaling is considered compromised and marked as “-“ when the expression of three or more AR regulated genes are reduced ($CI \leq 0$, Dobi et al., TOCJ, 2010).

These findings suggested different utility of detecting ARP genes by IHC when comparing to measuring mRNA levels. **Detection of ARP proteins may complement the clinical-pathologic features of higher grade tumors supporting the prediction of BCR. In contrast, detecting attenuated expression of ARP mRNA levels in index tumors with well-to-moderately differentiated morphology may support the prediction of favorable outcome.**

Encouraged by the promising data observed by the analysis of ARP proteins (Figure 7.), in Year #3-4 we have extended the ARP protein IHC analyses examining 80 whole-mounted FFPE radical prostatectomy specimens. We have focused this experiment to address ARP IHC performance in both CA and AA men leveraging the high representation of AA men enrolled to the CPDR's Biospecimen Bank and National Database. In this study we carefully matched patients by clinicopathologic features including time rane of RP. We have developed a numeric ARP IHC score (0-3) from the percentage and staining and intensity of index tumors. The numeric scores were further validated and refined to a three color IHC scale to represent ARP readout (Figure 8). With the exception of *ERG* mRNA detection in index tumors, frequency of attenuated mRNA expression of AR-regulated genes showed similat overall frequencies in AA or CA patients. The specificity of detecting attenuated ARP mRNA was 74% with a sensitivity of 53% in CA. *ERG* frequencies were shown to be significantly lower (28%) in AA men (Rosen et al., 2012) and only 17% in high grade tumors (Farrell et al., 2014). Therefore, in AA patients detection of ARP mRNA was less informative due to the high frequency of *ERG* negative index tumors.

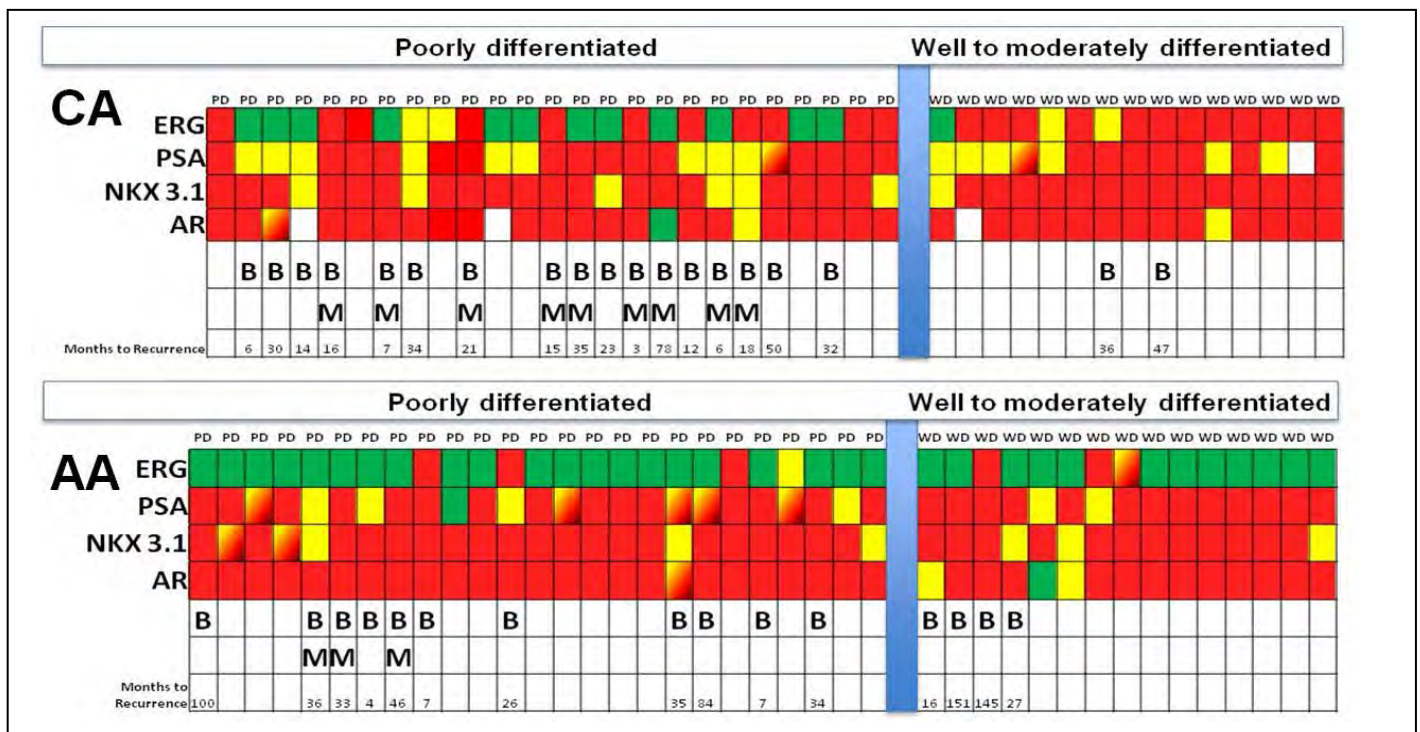


Figure 8. Performance of ARP protein detection by IHC in poorly differentiated and well to moderately differentiated index tumors in Caucasian American (CA) and African American (AA) Men. Follow up range: 10-17 years; B= BCR; M= Metastasis. ARFI scores: IHC color intensities and percentage of staining of index tumors were calculated in a numeric scale of 0-3 and were converted to color codes as follows: red: normal, positive >2.4 (80-100%); yellow: decreased 1.2-2.4 (40-80%); green: absent, negative <1.2 (0-40%). Specificity and sensitivity for adverse clinical features, such as BCR and/or metastasis within a 10-17 years of follow up were assessed. Decreased expression of PSA and NKX3.1 was consistently observed in index tumors of both AA and CA men with poorly differentiated index tumors. The overall pattern of AR regulated genes were similar except ERG, that was absent in majority of index tumors of AA men. In CA patients the ARP IHC score showed 74% specificity and 54% sensitivity for predicting BCR and or metastasis. In AA men 52% the specificity and 47% sensitivity was observed likely due to the high frequency of ERG negative tumors.

To evaluate the performance of ARP mRNA detection, in Year # 4 we have employed the quantitative technology platform, NanoString. The NanoString platform is suitable for the analyses of RNA isolated from FFPE tissues, probes can be multiplexed yielding absolute quantitation by counting the copy number of detected mRNAs. The expression levels of AR-regulated genes (*ERG*, *PSA/KLK3*, *PMEPA1*, *ODC1*, and *AMD1*) were determined in 63 macrodissected FFPE-derived tumor samples by NanoString platform. The study design was focused on low grade disease (Gleason 3+3 or 3+4; no positive margins, no extracapsular extension, and no seminal vesicle invasion) with (N= 21) or without (N=42) recurrence (BCR or distant Metastasis). NanoString analysis was performed on all 63 macrodissected tumor cDNA samples (Figure 9.).

Leveraging the observation that measuring mRNA levels of the ARP suggested better performance in predicting favorable outcome in low grade disease (Figure 7) we evaluated the prognostic potential of androgen regulated genes by the NanoString platform. **Analyses revealed that the ARP gene panel differentiates the BCR group from the BCR-free group with 74% specificity and 76%sensitivity.**

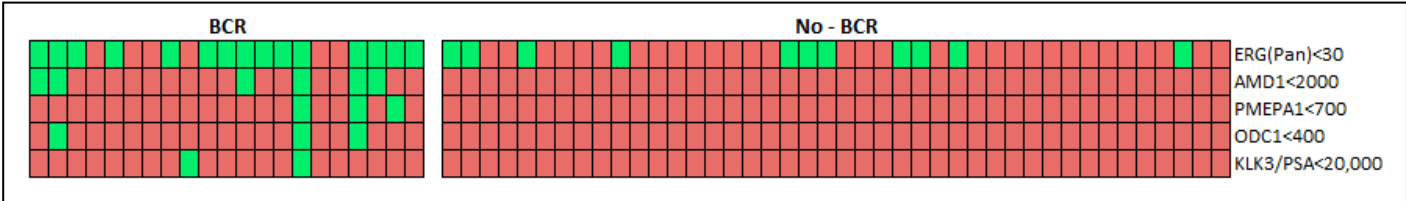


Figure 9. Assessment of ARFI gene expression in tumor mRNA samples of low grade disease indicates high specificity for confirming BCR-free survival. BCR group is marked by BCR or No-BCR in the heading. Copy number cutoff for BCR of *ERG*, *AMD1*, *PMEPA1*, *ODC1* and *KLK3*(*PSA*) mRNA is indicated on the right. Red color marks normal expression levels, green indicates decreased expression of ARFI genes below the copy number cutoff values in tumors.

The remarkable separation power of androgen regulated genes (*ERG*, *AMD1*, *PMEPA1*, *ODC1* and *PSA*) is shown by significantly lower frequency of attenuated ARP gene expression within the patient group with BCR-free survival (No-BCR) and by the frequent detection of decreased ARFI gene expression within the BCR group. Green in the heat map represents index tumors for each patient with gene expression below the cutoff value.

c) Other achievements:

We have completed a collaborative study with Dr. Lukas Bubendorf, University of Basel directly addressing the correlation of ERG protein expression (as the result of androgenic activations) and *ERG* gene rearrangements on the progression to castration resistant prostate cancer (CRPC) (Figure 10.). In this study design we examined tissue microarray from 114 hormone naïve and 117 CRPCs. We analyzed the expression of ERG oncoprotein by IHC and ERG rearrangement status by

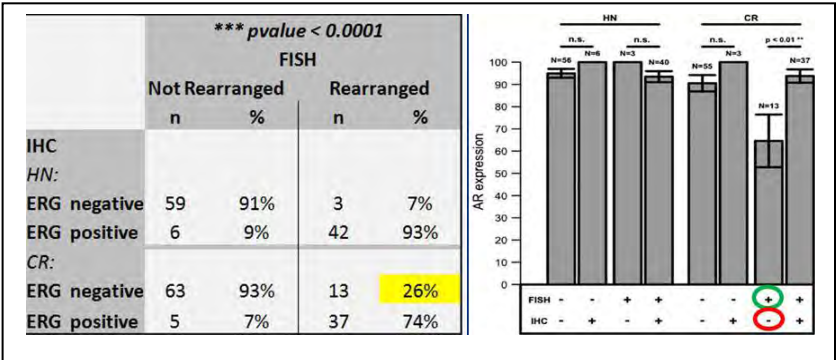


Figure 10. The presence of ERG fusions with absent ERG protein indicates a thus far unrecognized subset of CRPCs with dispensed AR pathway.

fluorescence in-situ hybridization. Also, we monitored the protein expression levels of AR and the proliferation marker Ki67. Consistent with previous reports the *TMPRSS2-ERG* gene fusion status showed correlation with the presence or absence of ERG protein both in hormone naïve and in CRPC specimens ($p < 0.0001$). **The major finding of the study is the complete absence of ERG protein in 26% of CRPC cases harboring ERG genomic rearrangement. Thus, presence of ERG fusions with absent ERG protein revealed a thus far unrecognized subset of CRPCs with dysregulated AR pathway who may not benefit from conventional therapy directed against the AR pathway (Gosper et al., PCPD 2014). This finding is consistent with the central hypothesis of the proposal.**

d) Opportunities for training and professional development

This project provided training opportunity to a 4th year Urology Resident, WRNMMC under the half year rotation to the CPDR Resident's Translation Research Training Program. The goal of the resident's research was to establish the numeric scoring system for the IHC staining intensities of AR genes and draft the manuscript and meeting abstracts towards the publication of findings.

4. IMPACT:

With the support of the PCRP/CDMRP/DoD Idea Development Award we investigated the expression levels of an AR regulated gene panel, AR to improve patient stratification for outcome prediction and for specific therapeutic approaches in prostate cancer treatment. **In our approach we selected genes leveraging the mechanistic understanding of direct AR regulated genes. Thus, our approach is distinct from the empirically designed OncotypeDX Prostate mRNA-based AR-regulated gene set that has been recently introduced to clinical practice by Genomic Health Inc.**

The major finding emerging from our PCRP/CDMRP award is that there is clear difference between the utility of AR protein detection and the detection of AR mRNA levels in prostate cancers.

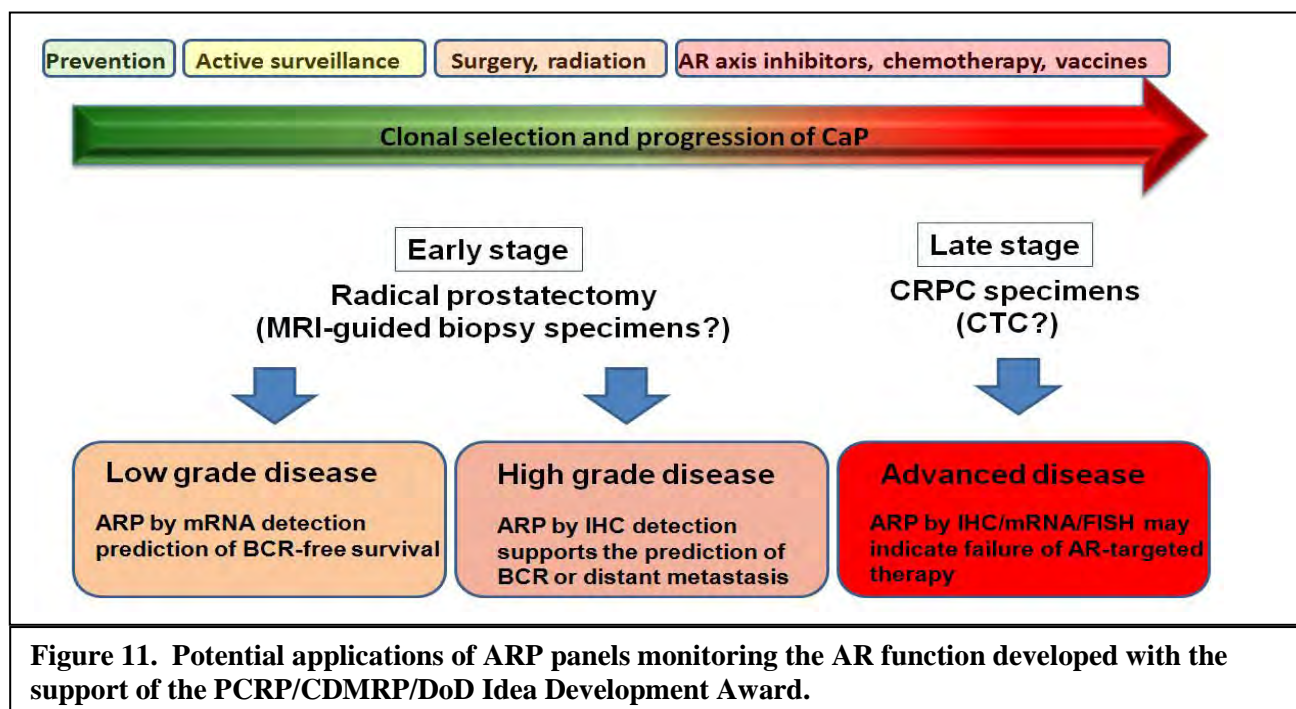
1) Monitoring AR function at protein expression levels of androgen regulated genes may provide more easily adaptable and cost effective IHC-based approach in routine pathology settings. The association of AR function defects, decreased or attenuated expression of AR proteins, showed 74% specificity in predicting BCR/distant metastasis in support of unfavorable clinical-pathologic features. This performance was demonstrated among CA patients.

2) Assessment of AR function at mRNA expression levels showed promising performance in evaluating low grade index tumors. Attenuated AR mRNA expression was frequently observed in patients who later experienced BCR. In contrast AR mRNA defect was rarely found in index tumors of patients with BCR-free survival yielding an overall 74% specificity and 76% sensitivity for BCR.

3) Examination of castration resistant prostate cancers our collaborative study revealed the absence of ERG protein in 26% of CRPC cases harboring *ERG* genomic rearrangement. Thus, presence of *ERG* fusions with absent ERG protein indicates a thus far unrecognized subset of CRPCs with dysregulated AR pathway who may not benefit from conventional therapy directed against the AR pathway.

In summary, consistent with the central hypothesis of this proposal the study results indicate the utility of monitoring the defects of AR function in prostate cancer. The observed readouts of AR dysfunction may

provide practical tool for improved prognostic accuracy and patient stratification to predict treatment response at early stages of prostate cancer (Figure 11).



5. CHANGES/PROBLEMS:

There were no changes to the original central hypothesis and specific aims. This award has been granted for a twelve month no cost extension by the grant agency Grant Officer with an approved SOW to solidify promising findings generated under this award focused on the evaluation of the AR gene panel towards defining predictive power and utility in patient stratification. The task of the comparative assessment of mRNA and protein ARP by branched-chain DNA (b-DNA) signal amplification method has been replaced by a comparison of microarray (Affymetrix GeneChip) derived tumor gene expression data set with IHC staining from matched tumor specimens. This change was due to limitations in the accuracy of branched-chain DNA (b-DNA) signal amplification method. The change had no impact on the overall progress of the proposal. Evaluating the mRNA expression levels of ARP genes we have completed the analysis of 77 LCM sample. To extend this task we have employed a new and more quantitative technology platform, NanoString. Initial data of ARFI mRNA performance in low grade prostate cancers suggests remarkable specificity for the prediction of BCR-free survival. Building on the encouraging results we are aiming to further develop the ARP panel to pre-clinical validation phase. Although, significant correlations were noted between attenuated expression of ARP and disease progression, converting the ARP readouts to nomogram was challenging due to the overall low number of examined cases and events. However, further effort to achieve this goal is warranted.

6. PRODUCTS:

a) Peer-reviewed articles

1) Gsponer JR, Braun M, Scheble VJ, Zellweger T, Rentsch CA, Bachmann A, Perner S, Sesterhenn IA, Srivastava S, Dobi A, Bubendorf L, Ruiz C.: **Analysis of the ERG rearrangement and protein expression in the progression from hormone-naïve to castration-resistant prostate cancer.** *Prostate Cancer and Prostatic Diseases* 17, 126-131 (2014).

Acknowledgement of PCRP/CDMRP/ DOD support: yes

2) Sharad S, Ravindranath L, Haffner MC, Li H, Yan W, Sesterhenn IA, Chen Y, Ali A, Srinivasan A, McLeod DG, Yegnasubramanian S, Srivastava S, Dobi A, Petrovics G.: **Methylation of the PMEPA1 gene, a negative regulator of the androgen receptor in prostate cancer.** *Epigenetics* 9, 918-927 (2014).

Acknowledgement of PCRP/CDMRP/ DOD support: yes

3) Invited article: Dobi A, Sreenath T, Srivastava S.: **Biological and Clinical Implications Androgen-responsive genes in prostate cancer:** Regulation function and clinical application (edited by Zhou Wang), Chapter 19: Androgen-dependent oncogenic activation of ETS transcription factors by recurrent gene fusions in prostate cancer: Springer Science & Business Media, LLC2013, BWF Book 300271, New York USA DOI 10.1007/978-1-4614-6182-1_19 (2013).

b) Podium presentation

Albert Dobi, Denise Young, Lakshmi Ravindranth, Wei Huang, Aaron Brothers, Shashwat Sharad, Hua Li, Gyorgy Petrovics, David G. McLeod, Isabell A. Sesterhenn and Shiv Srivastava: **Evaluation of Androgen Receptor Dysfunction to Enhance Stratification for Prostate Cancer Treatment.** American Urological Association (AUA) Annual Meeting, Hungarian-American Section, May 16-21, 2014, Orlando, FL

Acknowledgement of PCRP/CDMRP/ DOD support: yes

Resident's Competition: Aaron Brothers, Denise Young, Yongmei Chen, Gyorgy Petrovics, Jennifer Cullen, Inger L. Rosner, David G. McLeod, Albert Dobi, Shiv Srivastava, Isabell A. Sesterhenn: **Performance of the androgen receptor function index in African American and Caucasian American prostate cancers.**

Society of Government Service Urologist, 2015 Kimbrough Seminar, January 14-18, 2015, San Diego, CA

Acknowledgement of PCRP/CDMRP/ DOD support: yes

c) Moderated poster presentation

Albert Dobi, Denise Young, Lakshmi Ravindranth, Wei Huang, Aaron Brothers, Shashwat Sharad, Hua Li, Yongmei Chen, Gyorgy Petrovics, David G. McLeod, Isabell A. Sesterhenn, Shiv Srivastava: **Evaluation of androgen receptor function in prostate cancer.** *American Urological Association (AUA) Annual Meeting*, May 16-21, 2014, Orlando, FL

Acknowledgement of PCRP/CDMRP/ DOD support: yes

Aaron Brothers, Denise Young, Yongmei Chen, Gyorgy Petrovics, Jennifer Cullen, Inger L. Rosner¹, David G. McLeod, Albert Dobi, Shiv Srivastava, Isabell A. Sesterhenn : **Evaluation of androgen receptor function in African American and Caucasian American prostate cancers.** American Urological Association (AUA) Annual Meeting, May 15-19, 2015, New Orleans, LA

Acknowledgement of PCRP/CDMRP/ DOD support: yes

d) Poster presentations

Ruiz C, Braun M, Scheble VJ, Zellweger T, Rentsch CA, Bachmann A, Albert Dobi, Perner S, Sesterhenn I, Srivastava S, Bubendorf L.: **Comprehensive analysis of the TMPRSS2-ERG translocation during prostate cancer progression.** American Urological Association (AUA) Annual Meeting, Atlanta, GA, May 19 - 23, 2012.

Albert Dobi, Denise Young, Wei Huang, Lakshmi Ravindranath, Shashwat Sharad, Hua Li, Gyorgy Petrovics, David G. McLeod, Isabell A. Sesterhenn and Shiv Srivastava.: **Assessment of the Androgen Receptor Function Index (ARFI) in Prostate Cancer.** American Association for Cancer Research (AACR) Annual Meeting, April 6-10, 2013, Washington, DC

Albert Dobi, Gyorgy Petrovics, Denise Young, Wei Huang, Lakshmi Ravindranath, Shashwat Sharad, Yongmei Chen, Isabell A. Sesterhenn, David G. McLeod and Shiv Srivastava.: **Evaluation of the Functional Status of Androgen Receptor in Prostate Cancer.** 8th Early Detection Research Network (EDRN) Scientific Workshop, March 13-15, 2014, Bethesda, MD

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATION

There was no change in the active other support of the PD/PI(s) or senior/key personnel.

This award supported the employment and post-doctoral training of Shashwat Sharad, PhD. He has contributed to the qRT-PCR analysis of *PMEPA1*, *PSA* and *GAPDH* genes.

The Research Assistant position of this award, supported the salary of Ms. Wei Huang, MS., a full-time employee of CPDR. She has experience in key techniques pertinent to this proposal. Ms. Huang supported the completion of tasks in Aim #1 and Aim #2.

Biostatistician Yongmei Chen, MD, MPH. (5%) effort performed the analysis of clinic-pathology data correlations. Correlation analyses include the qRT-PCR and IHC data towards developing the ARP cumulative index.

4th year Urology Resident (no salary from this award), CPT Aaron Brothers, MD, WRNMMC was under the half year rotation to the CPDR Resident's Translation Research Training Program. The goal of the resident's research was to establish the numeric scoring system for the IHC staining intensities of ARP genes and draft the manuscript.

Collaborators: Lukas Bubendorf, MD, and Christian Ruiz PhD, from Institute for Pathology, University Hospital Basel, University of Basel, Switzerland collaborators performed the assessment of Castration Resistant Prostate Cancers. The PI and Co-PIs of the PCRP/CDMRP award provided study design, reagents, data analysis and interpretation.

8. SPECIAL REPORTING REQUIREMENTS

N/A

9. REFERENCES

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10. APPENDICES

(Attached)

ORIGINAL ARTICLE

ERG rearrangement and protein expression in the progression to castration-resistant prostate cancer

JR Gsponer¹, M Braun², VJ Scheble³, T Zellweger⁴, A Bachmann⁵, S Perner², T Vlainic¹, M Srivastava⁶, S-H Tan⁷, A Dobi⁷, IA Sesterhenn⁸, S Srivastava⁷, L Bubendorf¹ and C Ruiz¹

BACKGROUND: Approximately half of the prostate carcinomas are characterized by a chromosomal rearrangement fusing the androgen-regulated gene *TMPRSS2* to the oncogenic *ETS* transcription factor *ERG*. Aim of this study was to comprehensively analyze the role and impact of the ERG rearrangement and protein expression on the progression to castration-resistant (CR) disease.

METHODS: We used a tissue microarray (TMA) constructed from 114 hormone naive (HN) and 117 CR PCs. We analyzed the *ERG* rearrangement status by fluorescence *in situ* hybridization and the expression profiles of ERG, androgen receptor (AR) and the proliferation marker Ki67 by immunohistochemistry.

RESULTS: Nearly half of the PC tissue specimens (HN: 38%, CR: 46%) harbored a *TMPRSS2-ERG* gene fusion. HN PCs with positive translocation status showed increased tumor cell proliferation ($P < 0.05$). As expected, *TMPRSS2-ERG* gene fusion was strongly associated with increased ERG protein expression in HN and CR PCs (both $P < 0.0001$). Remarkably, the study revealed a subgroup (26%) of CR PCs with ERG rearrangement but without any detectable ERG protein expression. This subgroup showed significantly lower levels of AR protein expression and androgen-regulated serum PSA (both $P < 0.05$).

CONCLUSIONS: In this study, we identified a subgroup of *ERG*-rearranged CR PCs without detectable ERG protein expression. Our results suggest that this subgroup could represent CR PCs with a dispensed AR pathway. These tumors might represent a thus far unrecognized subset of patients with AR-independent CR PC who may not benefit from conventional therapy directed against the AR pathway.

Prostate Cancer and Prostatic Disease advance online publication, 28 January 2014; doi:10.1038/pcan.2013.62

Keywords: castration resistance; *TMPRSS2-ERG*; ERG

INTRODUCTION

Prostate cancer (PC) is the most frequently diagnosed cancer among males in western countries and the second leading cause of cancer-related death.¹ Although the mortality of PC has decreased mainly due to earlier detection, this disease still accounts for 9% of the total cancer deaths. Most PCs are nowadays diagnosed at an early stage. They initially depend on androgens for their growth and are thus referred to as hormone naive (HN) PC. Based on this dependence, the standard treatment for patients harboring these tumors is androgen-deprivation therapy (ADT). Although this therapy is initially effective, most of the treated tumors recur after a few months or years as castration-resistant (CR) PC. Mechanisms responsible for this progression are not fully understood.

PC research was revolutionized by the discovery of the *TMPRSS2-ERG* gene fusion in 2005.² Later on, it was realized that this rearrangement was part of a whole family of gene fusions that connect the promoter region of androgen-regulated genes, most frequently the *TMPRSS2* (transmembrane protease inhibitor 2) with transcription factors of the *ETS* (erythroblastosis virus E26 transforming sequence) family of transcription factors.^{3,4} Of these fusions, the rearrangement involving the genes *TMPRSS2* and *ERG*

is by far the most common (>90%) and is present in approximately 50% of prostate tumors.⁵ The two involved genes are <3 Mb apart on chromosome 21, and their fusion can occur through various rearrangements mechanisms, most frequently deletion of the intervening region on chromosome 21 (reviewed in Tomlins *et al.*⁶ and Perner *et al.*⁷). This rearrangement results in an androgen regulation of the *ERG* gene, leading to the overexpression of this gene in prostatic adenocarcinoma (reviewed in Sreenath *et al.*⁸). Despite the extensive studies about the role of the *ERG* rearrangement and expression, its clinical significance remains controversial.^{9,10} Recently, Minner *et al.*¹¹ did not observe any prognostic impact in a larger cohort of radically operated PCs.

In CR PC, *ERG* rearrangement has been shown to prevail in 34–45% of the tumors.^{12,13} Very recently, we observed a higher frequency of *ERG* rearrangements (45%) in recurrent CR PC specimens and a lower frequency of 25% in metastatic CR PCs.¹³ In contrast to the rearrangement, which is present on a genomic level, ERG protein expression is more dynamic, as it depends on the presence and activation of the androgen receptor (AR). In the CR disease state, the tumor may adapt to very low levels of androgens. Thus, it is not evident if these levels are sufficient for

¹Institute for Pathology, University Hospital Basel, University of Basel, Basel, Switzerland; ²Department of Prostate Cancer Research, Institute of Pathology, University Hospital of Bonn, Bonn, Germany; ³Institute of Pathology, Comprehensive Cancer Center, University Hospital Tuebingen, Tuebingen, Germany; ⁴Division of Urology, St. Claraspital, Basel, Switzerland; ⁵Department of Urology, University Hospital Basel, University of Basel, Basel, Switzerland; ⁶Department of Anatomy, Physiology, and Genetics, and Institute for Molecular Medicine, Uniformed Services University of the Health Sciences, School of Medicine, Bethesda, MD, USA; ⁷Center for Prostate Disease Research, Department of Surgery, Uniformed Services University of the Health Sciences, Bethesda, MD, USA and ⁸Joint Pathology Center, Silver Spring, MD, USA. Correspondence: Dr C Ruiz, Institute for Pathology, University Hospital Basel, University of Basel, Schönbeinstrasse 40, Basel 4031, Switzerland.

E-mail: christian.ruiz@unibas.ch

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the activation of ERG transcription. Data from these investigations have provided controversial results: whereas in some CR PC xenograft experiments ERG mRNA expression was not detectable,¹⁴ others have shown ERG protein expression in rearranged CR PC samples and xenografts.^{15,16}

In the present study, we used a tissue microarray (TMA) consisting of 231 locally advanced PCs that were collected either before (HN) or after recurrence to ADT (CR). We used this TMA to comprehensively interrogate and characterize the ERG protein expression and rearrangement comparing HN and CR PCs. We included standard markers into our analyses known to be relevant in PC, such as AR protein expression and Ki67 labeling. Here, we show that a considerable fraction of *ERG*-rearranged CR PCs loses ERG protein expression. We hypothesize that this might be due to a dispensed AR pathway.

MATERIALS AND METHODS

TMA and patients

The use of clinical specimens for the construction of the castration resistance TMA (crTMA) was approved by the ethical committee of the University and the University Hospital of Basel, Basel, Switzerland. The crTMA was manufactured as previously described.¹⁷ Briefly, tissue cylinders with a diameter of 0.6 mm were punched from the 'donor' tissue blocks containing the TURP specimens using a home-made, semi-automatic robotic precision instrument. Three cores from each specimen were arrayed. The composition of the crTMA has been previously described and is summarized in Supplementary Table S3.¹⁸ Briefly, it is composed of 697 spots from 231 TURPs from a total of 202 patients treated with advanced, locally obstructive PC. In addition, it contains 12 specimens from BPH. Castration resistance was defined as locally obstructive recurrence and/or PSA-recurrence during ADT.

Immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH)

IHC was performed according to the standard indirect immuno-peroxidase procedures. The primary antibody was omitted for negative controls. All slides were read manually by an experienced pathologist (LB). Data from AR and Ki67 were available from a previous study on the same TMA block.¹⁸ Briefly, the antibodies M3562 and M7240 (both DAKO, Carpinteria, CA, USA) were used for AR and Ki67 staining, respectively. The anti-ERG mouse monoclonal antibody 9FY was from Biocare Medical (Concord, CA, USA).¹⁹ FISH analysis for detection of *ERG* rearrangement was performed as previously reported.¹³ Images were obtained by usage of the AXIO Imager.A1 microscope equipped with an AxioCam and the AxioVision 4.6 software (all from Zeiss, Jena, Germany).

Cutoffs, data analysis and statistics

For protein expression analysis of AR, Ki67 and ERG, the percentage of positive tumor cells was noted by an experienced pathologist (LB) and used as score.¹⁸ For dichotomous stratification of ERG, samples with any specific positivity were considered as ERG positive (that is, cutoff > 0) and were considered negative in reference to endothelial ERG-positive staining.^{19,20} Cutoffs for definition of low or high for Ki67 labeling index were used as previously described.¹⁸ For correlation studies between different markers, every evaluable spot was considered for the analysis, that is, the analyses were performed on a 'spot-by-spot' basis. All other analyses (that is, descriptive tables, association with clinical data, such as treatment status, cM, cT and survival data) were performed on a 'one-value-per-biopsy' basis, thereby considering only one value per biopsy/specimen. If more than one spot/value per biopsy/specimen was evaluable, the spot with the maximal score was included in the analysis.

Statistical analysis was performed with the R Framework Version 3.0.1²¹ including the 'coin' package.²² Differences between two groups were analyzed with the Wilcoxon's rank-sum test; differences between more than two groups were analyzed using the Kruskal–Wallis rank-sum test for metric variables, for example, expression score. χ^2 and Fisher's exact test were used to analyze contingency tables. Survival curves were plotted by usage of the Kaplan–Meier method, and differences were assessed using the log-rank test. *P*-values < 0.05 were considered as statistically significant.

RESULTS

ERG expression and *TMPRSS2-ERG* rearrangement in HN and CR PC and association with clinicopathological features

To interrogate ERG protein expression and rearrangement by IHC and FISH, respectively, in the context of progression to castration resistance, we used the recently described crTMA that was constructed for this purpose.¹⁸ In addition, we included IHC data for AR and Ki67 expression from a previous study.¹⁸

For ERG expression analysis, 78 (68%) and 88 (77%) out of 114 HN and 117 CR TURPs, respectively, were evaluable (Figure 1). Of note, only cases with unequivocal nuclear staining for ERG in endothelial cells were considered as evaluable. ERG FISH analysis was successful in 94 (83%) and 94 (81%) of the 114 and 117 HN and CR PCs, respectively. ERG protein positivity, as well as the presence of ERG rearrangement, showed similar distributions between HN and CR PC (Table 1a). We found ERG protein positivity in 47% (37/78) and 40% (35/88) of the HN and CR PC samples. Similarly, 38% (36/94) and 47% (44/94) of the same samples showed ERG rearrangement. High-grade prostatic intraepithelial neoplasias were not present in this TMA and thus not analyzed in this study. We did not observe ERG positivity in the 10 evaluable BPH samples present on this TMA. In addition, the crTMA comprises a unique set of 36 matched PC samples from the same patients before (HN) and after hormonal ablation therapy (CR). The analysis of this subset revealed a change of ERG status in individual patients to be rare (1/21 and 2/30 for IHC and FISH, respectively; Supplementary Table S1).

We next investigated a potential association between ERG status and clinicopathological features, such as cM and cT stages, and Gleason pattern. ERG status was not differentially distributed across different cM and cT stages (data not shown). Interestingly, only ERG protein expression but not *ERG* rearrangement revealed a significant decrease of positivity toward higher Gleason pattern. This was true in HN ($P=0.004$) as well as in CR PCs ($P=0.019$) (Table 1b). As PCs of higher Gleason pattern are characterized by higher tumor cell proliferation, we investigated a potential correlation between ERG status and Ki67 labeling index. We did not observe a correlation between ERG protein expression and increased tumor cell proliferation. This was also true for *ERG* rearrangement. However, stratification into HN and CR revealed that the proliferation index in *ERG*-rearranged HN was significantly higher than in those HN where *ERG* was not rearranged (55% vs 38%, $P<0.05$, Supplementary Table S2).

No significant association of ERG status with overall survival of HN or CR PC patients

We analyzed the potential impact of ERG protein expression and rearrangement on overall survival. In both cohorts, HN as well as CR, neither ERG staining nor *ERG* rearrangement were related to patient prognosis in Kaplan–Meier survival analysis (Supplementary Figure S1).

Decreasing correlation of *TMPRSS2-ERG* translocation with protein expression of ERG in CR PC

It is well established that ERG protein expression is dependent on the presence of an *ERG* rearrangement in prostatic adenocarcinoma. Here we investigated the power of this correlation in the cohort of the crTMA, which is composed of highly advanced PCs before (HN) and after ADT (CR). As expected, a high correlation between *ERG* rearrangement and ERG protein expression was observed ($P<0.0001$). This was also true if PC samples were stratified according to their hormonal treatment status HN and CR ($P<0.0001$, Table 2). Intriguingly, whereas in HN PCs, the number of FISH-IHC discordant results were minimal (7% FISH positive, but ERG negative and 9% FISH negative, but ERG positive), in CR PCs, 26% (13 spots) of the *ERG*-rearranged samples did not show detectable ERG protein expression (Table 2). This surprisingly large

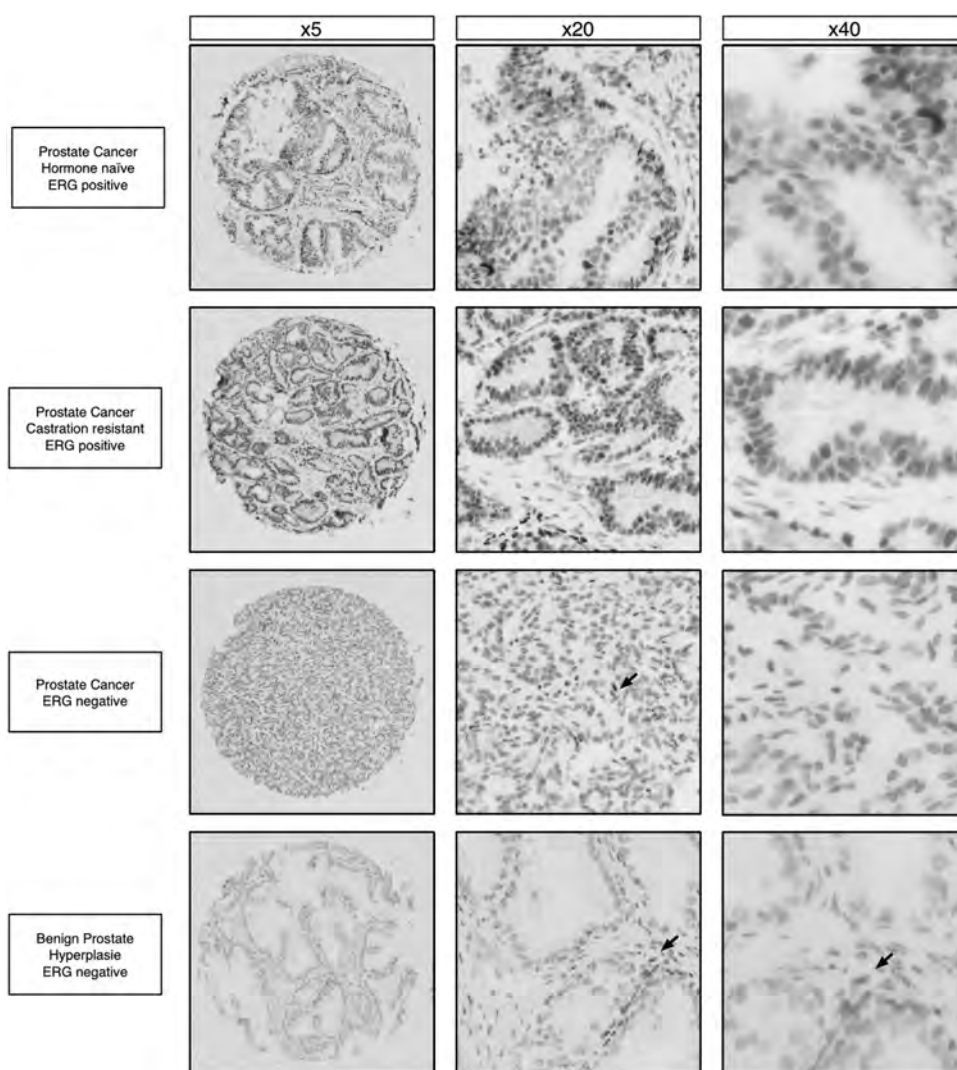


Figure 1. Representative images of ERG-stained prostate samples from the castration resistance tissue microarray (crTMA). Endothelial cells (black arrows) were used as positive control for the ERG staining.

group of *ERG*-rearrangement positive, but *ERG*-protein-negative PC samples in the CR, but not in the HN group, can hardly be explained by a technical phenomenon. These findings rather suggest that losing the high concordance between *ERG* FISH and *ERG* IHC toward more advanced PC samples may be due to the existence of a specific subset of CR PC patients whose tumors have lost the ability of expressing the *ERG* protein despite the presence of an *ERG* rearrangement. Of note, these 13 spots were from 11 different TURPs from 10 distinct patients.

TMPS2-*ERG*-positive CR PCs without detectable *ERG* protein expression

We next interrogated the association between the AR protein expression and the *ERG* status. As previously described,¹⁸ AR protein expression was present in almost all analyzed PC samples and maximal (score = 100) in > 90% of the specimens. Overall, we were not able to see a significant association between *ERG* rearrangement or positivity and AR expression ($P > 0.05$ both, data not shown). To analyze whether the *ERG* FISH vs IHC discrepancy in CR PC with *ERG* rearrangement but absent *ERG* protein is due to a loss of AR, we stratified the PCs into the different *ERG* subgroups according to the two treatment status. Although AR expression was present at high levels (score 90–100) in almost all PC samples,

independent of the *ERG* status, only *ERG*-rearrangement-positive CR PCs with absent *ERG* protein were characterized by lower levels of AR protein ($P = 0.002$, Figure 2a). Further, we interrogated a correlation of *ERG* protein expression with serum protein levels of the AR target gene PSA in the subgroup of *ERG*-rearranged CR PCs. As expected, the group of *ERG*-rearrangement positive and *ERG*-protein-negative CR PC samples had lower PSA levels than *ERG*-rearranged- and *ERG*-protein-positive samples ($P < 0.05$, Figure 2b). However, it must be considered that PSA serum information was only available for four *ERG*-rearrangement-positive but *ERG*-protein-negative CR PC samples.

DISCUSSION

The rearrangement of the *ERG* gene² and its associated expression in PC²³ has been the subject of numerous studies. Depending on the cohort used, the prevalence of the rearrangement and protein expression varies extensively (15–80%).¹³ Most of the studies have focused on the analysis of material from surgically resected prostates. In this study, we interrogated the *ERG* status on gene and protein levels in TURP specimens originating from HN and CR prostate tumors. For this purpose, we used a TMA specifically constructed for the analysis of disease progression.¹⁸

Table 1. Overview of the ERG status on the castration resistance tissue microarray (crTMA)

(a)									
		<i>BPH</i>		<i>All PC</i>		<i>NS</i>			
						<i>HN</i>		<i>CR</i>	
		n	%	n	%	n	%	n	%
<i>FISH</i>									
Not rearranged		10	100	108	57	58	62	50	53
Rearranged		0	0	80	43	36	38	44	47
<i>Immunohistochemistry</i>									
ERG negative		10	100	94	57	41	53	53	60
ERG positive		0	0	72	43	37	47	35	40
(b)									
<i>Gleason Pattern</i>		<i>NS</i>				<i>*P-value < 0.05</i>			
		<i>FISH</i>				<i>Immunohistochemistry</i>			
		Not rearranged		Rearranged		ERG negative		ERG positive	
		n	%	n	%	n	%	n	%
<i>HN</i>									
3		7	58	5	42	1	14	6	86
4		45	58	33	42	27	44	34	56
5		61	70	26	30	44	67	22	33
<i>CR</i>									
3		0	—	0	—	0	—	0	—
4		21	54	18	46	17	49	18	51
5		80	58	58	42	90	70	39	30

Abbreviations: CR, castration resistant; FISH, fluorescence *in situ* hybridization; HN, hormone naive; NS, not significant; PC, prostate cancer.

(a) ERG status was significantly different between BPH and all PCs, but not between HN and CR. Fisher's exact tests were used for comparisons.

(b) HN and CR prostate cancer samples without ERG protein expression are characterized by higher Gleason pattern. This association was not true for ERG rearrangement. The χ^2 test was used for comparison between the groups: Not rearranged vs rearranged and ERG + vs ERG – in HN samples. Fisher's exact test was used for CR samples.

Table 2. Correlation of ERG rearrangement and protein expression

***P-value < 0.0001				
FISH				
Not rearranged		Rearranged		
	n	%	n	%
Immunohistochemistry				
HN				
ERG negative	59	91	3	7
ERG positive	6	9	42	93
CR				
ERG negative	63	93	13	26
ERG positive	5	7	37	74

Abbreviations: CR, castration resistant; FISH, fluorescence *in situ* hybridization; HN, hormone naive.

A highly significant correlation was found between ERG rearrangement and ERG protein expression in each of the subgroups. ERG FISH-positive CR prostate cancers showed by far the highest discordant rate (26%). Analyses were performed on a spot level by usage of the Fisher's exact test.

We observed an overall ERG positivity rate of 43% of both ERG rearrangement and IHC positivity across all PC samples. This is similar to recent reports that found ERG protein positivity in 47% and 52% of the PC samples^{11,24,25} and ERG rearrangement in 47–55%.^{11,26,27} Stratification into HN and CR PC revealed a broader range (38–47%), but no significant differential positivity between these two groups could be detected. Concordantly, in the matched patient cohort, virtually all of the patients retained their ERG status after recurring under ADT. Although earlier reports that had focused on ERG RNA expression analysis or were based on tissues from xenografts had reported controversial prevalence rates in CR PC,²⁸ our findings are in line with a very recent study by Teng *et al.*²⁹ in which the authors observed the ERG expression in 37% of human CR PCs. These data strongly suggest that even lower levels of circulating androgens, as is the case under ADT therapy in patients with CR disease, are sufficient to sustain ERG expression in ERG-rearranged PC. Although no correlation of ERG status with clinico-pathological features, such as cM or cT stage, was found, we observed that at least for the protein expression, positive ERG status was associated with lower Gleason pattern (Table 1b). Of note, this TMA was not tailored for the analysis of the Gleason pattern, as most (97%) of the arrayed PCs show a high Gleason pattern (4 or 5) (Supplementary Table S3). In previous studies, TMPRSS2-ERG-negative PCs have already been associated to the highest

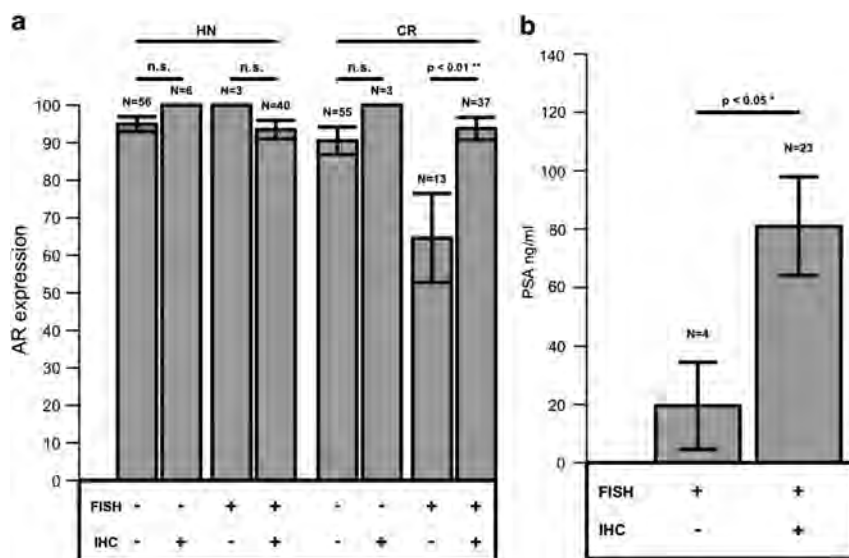


Figure 2. Differential androgen receptor (AR) and PSA protein expression in ERG-rearranged castration-resistant (CR) prostate cancer (PC). **(a)** AR protein expression in hormone naive (HN) and CR PC. Only ERG-rearranged CR PCs without ERG expression (discordant samples) showed significantly lower levels of AR protein expression. **(b)** Serum PSA levels in ERG-rearranged CR PC. The discordant samples (see Figure 2a) showed reduced levels of serum PSA. Statistical test used: Wilcoxon rank sum. n.s., not significant.

Gleason category studied.^{11,30} Similarly, we observed that a high fraction of tumors with Gleason Pattern 5 is ERG negative: 67% and 70% for HN and CR PCs by IHC, respectively, as well as 70% for HN PC by FISH (Table 1b). Interestingly, the lower number of ERG FISH-negative CR PCs with Gleason Pattern 5 (58%) might be explained by the higher number of ERG-discrepant CR PC samples in this study (see below).

As expected, we found a strong correlation between genomic rearrangement and protein expression in HN as well as in CR PCs, confirming that ERG expression depends on the presence of the *ERG* rearrangement, also in more advanced CR PCs. Stratification into the four different FISH (negative/positive) and disease (HN/CR) subgroups revealed that in the subgroup of *ERG*-rearranged CR PC the rate of discordant samples was surprisingly high (26%), suggesting that every fourth *ERG*-rearranged CR PC will no longer express the ERG protein. As the discordance rates in the other three groups were between 7% and 9%, the high discordance rate of 26% might be attributed to the defects of androgen signaling. Very recently, Teng *et al.*²⁹ had also reported a decrease in the consistency rate in the group of CR PC. However, their detected decrease was mainly due to *ERG* rearrangement negativity that needs to be further explored. Here, our findings suggest that up to 26% of the *ERG*-rearranged CR PC have lost their ability to express the ERG protein. These findings are consistent with a defective AR pathway.^{31,32} Indeed, only the discrepant samples of this subgroup (CR PC, *ERG* FISH positive) had significant lower levels of AR protein expression. Concordantly, this minor group of samples also had lower serum PSA levels. One could hypothesize that such patients with PCs who do not express androgen-responsive genes any longer might not be good candidates for a continuing ADT. However, it must be considered that serum PSA level information was only available for four patients of the subgroup of *ERG*-rearranged but *ERG*-protein-negative CR PCs. In a recently published study, we reported a subgroup of advanced CR PC patients whose tumors were characterized by the lack of AR expression and had a worst overall survival.¹⁸ Half of those tumors were classified as neuroendocrine prostate tumors, suggesting that they had circumvented AR dependency possibly by neuroendocrine-responsive trans-differentiation mechanisms.³³ In contrast, in the subgroup of *ERG*-discrepant samples (CR PC,

ERG FISH positive but IHC negative), only four out of the 13 stained positive for neuroendocrine markers, thus suggesting that neuroendocrine trans-differentiation alone cannot explain the characteristics of this subgroup. The four poorly differentiated neuroendocrine CR PCs included two small cell prostate carcinomas and two large cell neuroendocrine carcinomas. Further studies are needed to investigate the specific characteristics of this *ERG* FISH-positive but *ERG* IHC-negative subset of PCs on a molecular level and to define the role of *ERG* rearrangement and expression.

A limitation of our study is that our cohort comprises locally advanced and obstructive tumors from palliative TURPs. Materials from TURPs for TMA construction must be rigorously examined before construction to exclude areas with technical artifacts originating from the resection procedure (for example, heat/mechanical damage). However, PC specimens from these TURPs represent very valuable tissue samples, especially in the context of hormonal ablation. In this study, the stratification into different disease states (HN/CR) and FISH positivity groups limited the sample number in the different subgroups. Thus, studies with larger cohorts of HN and CR PC samples from TURPs are needed to further assess these findings and to evaluate the AR-downstream signaling pathways in the distinct HN/CR *ERG* subgroups. Despite these limitations, in this study we were able to show the prevalence of *ERG* positivity in HN and CR PC and that this positivity is not differentially distributed between these two disease groups. Importantly, we provide evidence for the existence of an *ERG*-rearranged PC subset of cases that has apparently lost the ability to express androgen-regulated genes.

CONFLICT OF INTEREST

The Henry M. Jackson Foundation for the Advancement of Military Medicine has filed a patent application on the mouse monoclonal anti-*ERG* antibody, 9FY, on which ST, AD and SS are co-inventors and has been licensed to the Biocare Medical. This study was conducted independent of any involvement from Biocare Medical. The Brigham and Women's Hospital and the University of Michigan have filed a patent on *ETS* gene rearrangements in prostate cancer, on which SP is a co-inventor and the diagnostic field of use has been licensed to GenProbe. GenProbe has not played a role in the design and conduct of the study, in the collection, analysis or interpretation of the data and had no involvement in the preparation, review or approval of the manuscript. All the other authors declare no conflict of interest.

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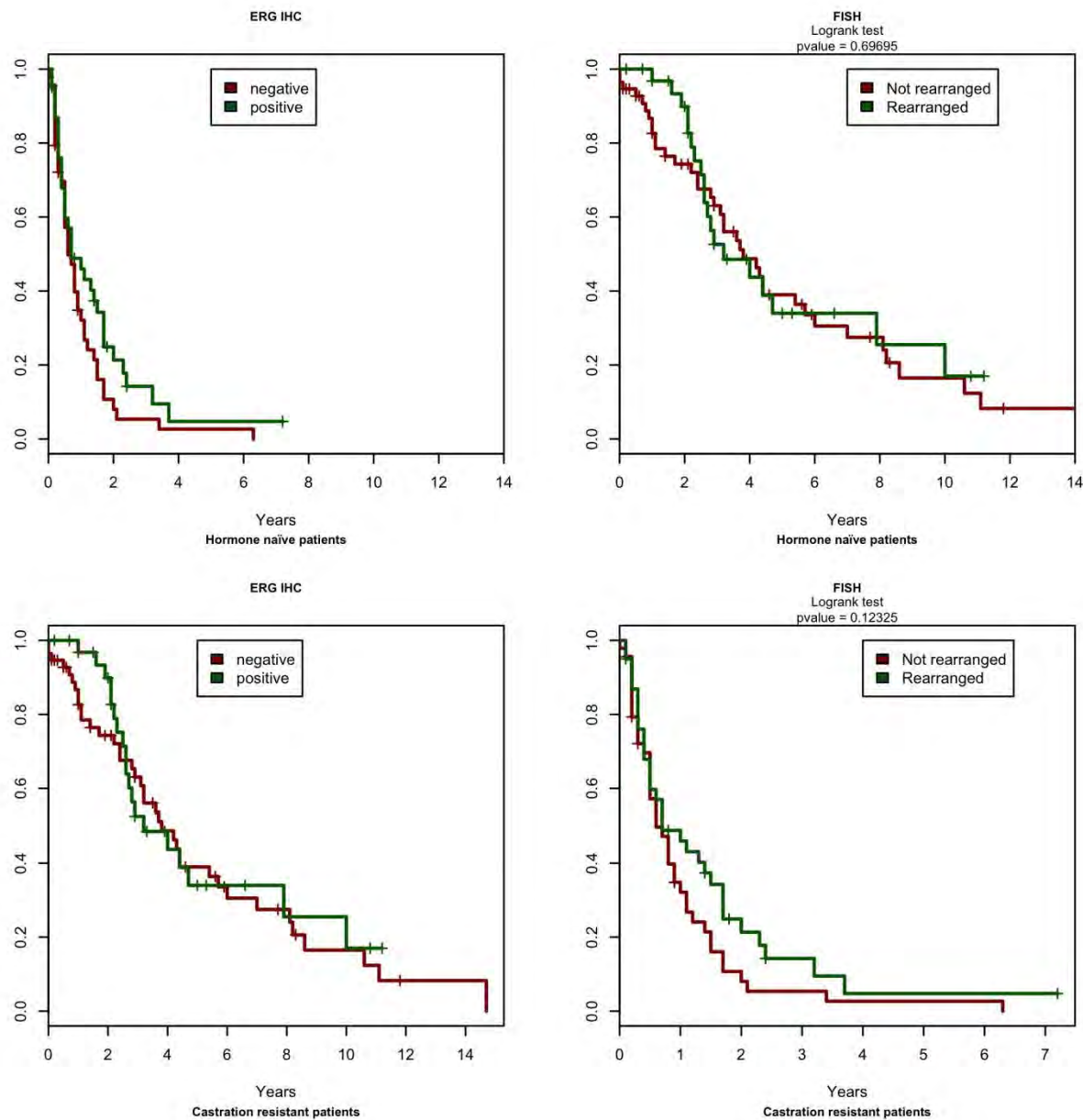
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Supplementary Information accompanies the paper on the Prostate Cancer and Prostatic Diseases website (<http://www.nature.com/pcan>)

Supplemental information

Supplemental Figure 1. Overall survival of HN and CR PC patients. No significant association of ERG status with overall survival for HN (upper panels) or CR (lower panels) PC patients were observed.



Supplemental Table 1. ERG status in matched HN/CR patient cohort. Nearly all (IHC: 20/21, FISH: 28/30) of the PC samples retained their ERG status after recurrence to castration resistance. *IHC: immunohistochemistry, FISH: fluorescence in-situ hybridization.*

Technique	Status in		N (%)
	HN	CR	
IHC	Negative	Negative	11 (52%)
	Positive	Positive	9 (43%)
	Negative	Positive	0
	Positive	Negative	1 (5%)
FISH	Negative	Negative	15 (50%)
	Positive	Positive	13 (43%)
	Negative	Positive	1(3%)
	Positive	Negative	1 (3%)

Supplemental Table 2. Correlation of ERG status with tumor cell proliferation. Significant association between ERG status and Ki67 LI (tumor cell proliferation) was only observed for FISH in HN PC. Fisher's exact tests were used for comparisons. *HN: hormone naïve, CR: castration resistant.*

	<i>HN: * pvalue < 0.05; CR: n.s.</i>				n.s.			
	FISH				Immunohistochemistry			
	Not Rearranged		Rearranged		ERG negative		ERG positive	
Ki67	n	%	n	%	n	%	n	%
<i>HN:</i>								
Low	64	##	27	45%	41	59%	32	55%
High	39	##	33	55%	28	41%	26	45%
<i>CR:</i>								
low	36	##	31	38%	37	40%	26	43%
high	50	##	50	62%	56	60%	35	57%

Supplemental Table 3. Overview of the cohort analyzed on this TMA. Summary of the HN and CR PC specimens on the crTMA used.

	Surgical Specimens		Spots on TMA	
<i>BPH</i>	12		36	
<i>Hormone naïve PC</i>	114		340	
<i>CRPC</i>	117		357	
	Hormone naïve	Castration resistant	Hormone naïve	Castration resistant
<i>Age at surgery</i>	Mean = 77.5 Median = 78.4 Min = 43.7 Max = 99.46		Mean = 77.7 Median = 79.8 Min = 49.8 Max = 90.9	
<i>Gleason pattern</i>			3: 19 4: 140 5: 145 na: 36	3: 1 4: 73 5: 256 na: 27
<i>cT</i>	1: 9 2: 8 3: 53 4: 26 na: 18	1: 1 2: 3 3: 22 4: 82 na: 8		
<i>cM</i>	0: 69 1: 27 na: 18	0: 31 1: 67 na: 18		
<i>Surgery to death</i>	Median = 3.8 (± 2.9 -5.4) years Records = 109 Events = 68		Median = 0.9 (± 0.7 - 1.2) years Records = 110 Events = 88	

Methylation of the *PMEPA1* gene, a negative regulator of the androgen receptor in prostate cancer

Shashwat Sharad¹, Lakshmi Ravindranath¹, Michael C Haffner², Hua Li¹, Wusheng Yan¹, Isabell A Sesterhenn³, Yongmei Chen¹, Amina Ali^{1,4}, Alagarsamy Srinivasan¹, David G McLeod^{1,4}, Srinivasan Yegnasubramanian², Shiv Srivastava¹, Albert Dobi^{1,*}, and Gyorgy Petrovics^{1,*}

¹Center for Prostate Disease Research; Department of Surgery; Uniformed Services University of the Health Sciences; Bethesda, MD USA;

²Sidney Kimmel Comprehensive Cancer Center; Johns Hopkins University; Baltimore, MD USA; ³The Joint Pathology Center; Silver Spring, MD USA;

⁴Urology Service; Walter Reed National Military Medical Center; Bethesda, MD USA

Keywords: *PMEPA1*, methylation, androgen receptor, tumor suppressor, prostate cancer, laser capture microdissection

Abbreviations: *PMEPA1*, prostate transmembrane protein androgen induced 1; AR, androgen receptor; CaP, cancer of the prostate; CA, Caucasian American; AA, African American; LCM, laser capture microdissection; SMAD, Mothers against decapentaplegic homolog; TGF- β , transforming growth factor-beta; PI3K, phosphatidylinositol-3-kinase

The prostate transmembrane protein androgen induced 1 (*PMEPA1*) gene is highly expressed in prostate epithelial cells and is a direct transcriptional target for the androgen receptor (AR). AR protein levels are controlled by the AR-*PMEPA1* negative feedback loop through NEDD4-E3 ligase. Reduced expression of *PMEPA1* observed in prostate tumors, suggests that loss of *PMEPA1* may play critical roles in prostate tumorigenesis. This study focuses on epigenetic mechanisms of reduced *PMEPA1* expression in the cancer of the prostate (CaP). Benign (n = 77) and matched malignant (n = 77) prostate epithelial cells were laser capture micro-dissected from optimum cutting temperature embedded frozen prostate sections from 42 Caucasian American (CA) and 35 African American (AA) cases. Purified DNA specimens were analyzed for CpG methylation of the *PMEPA1* gene. *PMEPA1* mRNA expression levels were evaluated by qRT-PCR. Analysis of *PMEPA1* methylation and mRNA expression in the same tumor cell populations indicated a significant inverse correlation between mRNA expression and methylation in CaP ($P = 0.0115$). We noted higher frequency of CpG methylation within the evaluated first intronic region of the *PMEPA1* gene in prostate tumors of CA men as compared with AA. In CaP cell lines, *PMEPA1* expression was induced and AR protein levels were diminished in response to treatment with the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (decitabine). Cell culture-based studies demonstrated that decitabine restores *PMEPA1* expression in AR-positive CaP cell lines. This report reveals the potential role of *PMEPA1* gene methylation in the regulation of AR stability. Thus, downregulation of *PMEPA1* may result in increased AR protein levels and function in CaP cells, contributing to prostate tumorigenesis.

Introduction

Androgens and the androgen receptor (AR) play central roles in the normal growth, differentiation and physiological function of the prostate gland.¹ It has also been established that AR dysregulation leads to the progression of cancer of the prostate (CaP).^{2–4} To suppress androgen-dependent CaP growth, currently used therapeutic agents inhibit the binding of androgens to AR or the biosynthesis of androgens.^{5–8} Although most CaP initially respond to androgen ablation, its therapeutic effect is short lived and patients eventually develop castration resistant CaP.^{5–9}

The AR binds to AR-responsive elements (AREs) and regulates the transcription of androgen responsive genes controlling

differentiation and growth.^{1–4} Dysfunction of the androgen axis contributes to CaP through numerous mechanisms, including increased AR expression, intra-tumoral androgen synthesis, AR splice variants, mutations of the AR and androgen metabolizing enzymes.^{2–9} In a subset of advanced CaP, AR signaling is bypassed in favor of AR independent pathways.^{2,3,10,11}

The *PMEPA1* gene was identified in our laboratory as a highly androgen-induced gene in a screen for androgen regulated genes in CaP cells.^{12,13} *PMEPA1* is predominantly expressed in the prostate gland and is directly regulated by AR.¹⁴ *PMEPA1* spans 55–60 kb on chromosome 20 (20q13.31-q13.33) and the *PMEPA1* protein is highly conserved among vertebrates, suggesting a critical role in the homeostasis of prostate. Functional

*Correspondence to: Gyorgy Petrovics; Email: gpetrovics@cpdr.org; Albert Dobi; Email: adobi@cpdr.org
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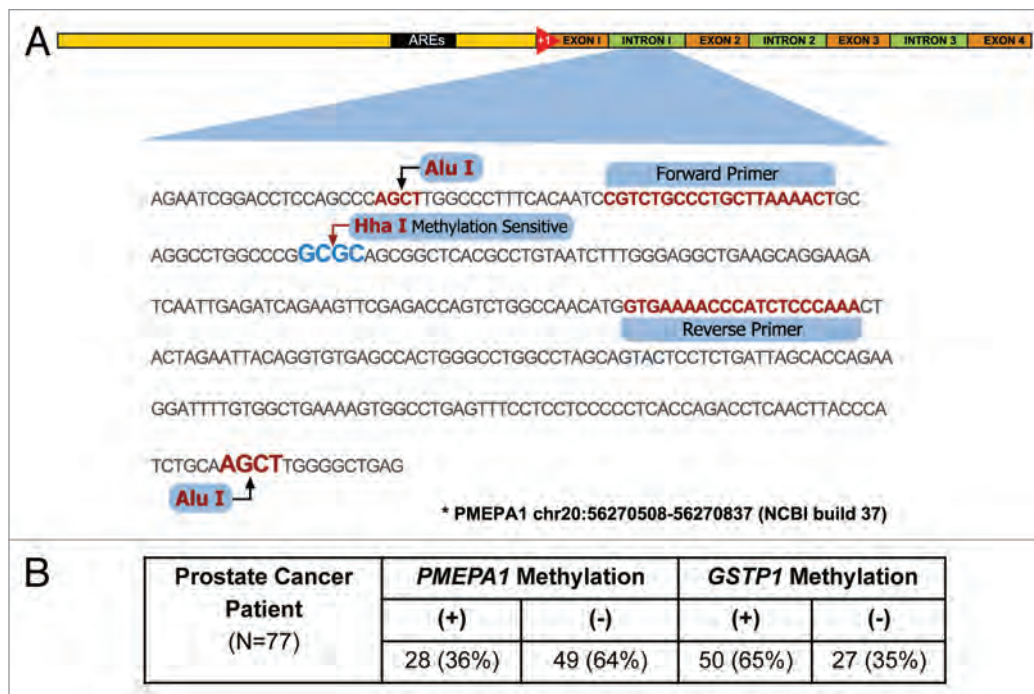


Figure 1. (A) Schematic representation of the *PMEPA1* gene structure. Red triangle (+1) marks the transcription initiation site. DNA methylation site (HhaI), PCR primer positions and the sequence of the assayed region of the *PMEPA1* gene. **(B)** Methylation frequencies of *PMEPA1* and *GSTP1* gene in LCM derived human prostate tumor cells from 77 patients.

analysis of *PMEPA1* has revealed that it is a NEDD4 E3-ligase binding protein and plays a role in downregulation of AR through a negative feedback loop between AR and *PMPEA1*.¹⁵ Inhibition of *PMEPA1* leads to increased AR levels in CaP cells. Thus, decrease or loss of *PMEPA1* mRNA expression that is frequently observed in CaP may result in gain of AR function.^{15,16} Studies also suggest that *PMEPA1* is involved in other cancers through regulation of the TGF- β , PI3K and WNT pathways.¹⁷⁻²⁰ These findings highlight the cellular context-dependent role of *PMEPA1* in normal and malignant conditions.

Activation of *PMEPA1* transcription by decitabine in LNCaP and LAPC4 cells with demethylation of CpG residues within the *PMEPA1* promoter downstream sequences suggested a role for DNA methylation in regulating *PMEPA1* in CaP.²¹ The current study focuses on the evaluation of the methylation and expression of the *PMEPA1* gene in primary prostate tumors and in CaP cell culture models. The results underscore the role of DNA methylation in silencing the *PMEPA1* gene with potential implications in the AR degradation pathway.

Results

PMEPA1 is frequently methylated in prostate cancer

We evaluated the methylation of the *PMEPA1* gene in human prostate tumors. As a positive control, we analyzed the promoter methylation of *GSTP1*, a gene known to be methylated in the majority of CaPs.²² Laser capture microdissection (LCM) was used for the precise isolation of tumor and matched non-adjacent

normal cells from 77 CaP cases. Genomic DNA and total RNA were isolated from each specimen.

We optimized the methylated-DNA precipitation and methylation-sensitive restriction enzymes (COMPARE-MS)²³ assay for 2 ng of purified genomic DNA obtained from LCM-dissected tumor cells. Methylation analysis was performed for CpG-rich sequences of *PMEPA1* gene (20q13.31-q13.33) (Fig. 1A). Additionally we analyzed *GSTP1* gene (11q13)²³ that is highly methylated in CaP²² and a *LINE1* repetitive DNA element that has been shown to harbor methylation in the human genome.²⁴

The cohort (42 CA and 35 AA patients) was designed to address the frequency of *PMEPA1* methylation. *PMEPA1* gene methylation was observed in 28 of 77 cases (36%), whereas *GSTP1* methylation was detected in 50 of 77 (65%) cases (Fig. 1B). As expected *GSTP1* methylation was highly prevalent. In this study, the cancer cells were isolated by LCM yielding low amounts of DNA suitable only for COMPARE-MS, an assay that has been shown to robustly enrich for methylated DNA with high sensitivity and specificity.²³ However, limitations in the sensitivity to detect methylated alleles are expected with low amount of DNA input DNA from LCM microdissected primary tumor cells. This likely explains the slightly lower rate of *GSTP1* methylation observed in this study compared with previously published studies.^{22,25}

The observed methylation of *PMEPA1* prompted us to investigate whether methylation affected the expression level of *PMEPA1* in CaP. Thus, we evaluated the level of *PMEPA1* gene expression from the same tumor samples that were evaluated for DNA methylation. Patient matched non-adjacent normal epithelial cells

were also analyzed for quantitative *PMEPA1* expression (n = 77). Matched tumor over normal relative *PMEPA1* expressions are summarized in **Figure 2A**. Consistent with our previous report,¹⁵ *PMEPA1* expression was reduced in two-thirds of CaP cases. Both Caucasian American (CA) (65%) and African American (AA) (62.9%) groups showed similar frequencies of decreased *PMEPA1* mRNA expression in CaP cells in comparison to matched normal epithelial cells.

Overall, methylation of the *PMEPA1* gene significantly correlated with reduced mRNA expression ($P = 0.0115$) (**Fig. 2B**). The analysis revealed that 82% of patients, who have methylated *PMEPA1* gene, have lower *PMEPA1* expression in tumors compared with matched normal epithelium (**Fig. 2B**). Taken together these findings suggest that DNA methylation plays major roles in silencing the *PMEPA1* in CaP. In this study, assessment of clinico-pathological data (**Table 1**) did not reveal correlation of biochemical recurrence with *PMEPA1* methylation status or expression that is likely due to the limited sample size.

Differential *PMEPA1* methylation between CA and AA patients

An unexpected findings of this study relates to differential methylation of *PMEPA1* between the CA and AA CaP ($P = 0.0064$) despite similar patterns of *PMEPA1* mRNA expression. These data suggested for additional mechanisms involved in downregulation of *PMEPA1* in AA CaP.

We noted a higher frequency of *PMEPA1* methylation (50%) in CA CaP in comparison to AA (20%) (**Fig. 3A and B**). The difference in *PMEPA1* methylation status between these two groups remained striking (CA: 57%; AA: 17%, $P = 0.0014$) even when only tumor cells with well-differentiated morphology were compared (**Fig. 3C**). In contrast, the methylation status of the *GSTP1* gene showed no significant difference (69% in CA and 60% in AA) (**Fig. 3A and B**). In the current study design the patient cohort represented nearly equal number of AA and CA cases. While both clinical (2.5-fold higher mortality in AA CaP patients) and cancer genome differences (low frequency of *TMPRSS2-ERG* in AA patients) have been consistently noted in literature between CA and AA patients,²⁶⁻²⁸ additional evaluations of differential *PMEPA1* methylation between CA and AA are warranted.

Association of *PMEPA1* methylation with AR in prostate cancer cell lines

Association of *PMEPA1* methylation with reduced expression of *PMEPA1* in CaP specimens provided a rational for testing the methylation status of *PMEPA1* in CaP cell lines. We used AR-positive (VCaP, LNCaP, and LAPC4) and AR-negative (DU145 and PC3) cell culture models to analyze the methylation of the *PMEPA1* gene. *GSTP1* methylation was monitored as an

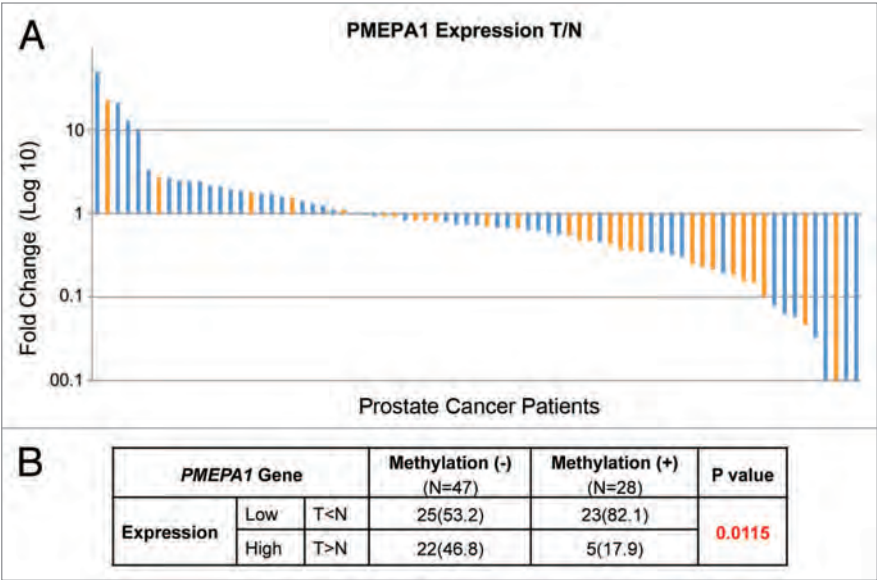


Figure 2. (A) Quantitative RT-PCR expression analysis of *PMEPA1* transcript in LCM derived paired normal and tumor cells of primary CaP revealed decreased expression of *PMEPA1* in two-third of CaP patients (Log 10 tumor/normal [T/N]). Cases (tumors) with methylated *PMEPA1* gene are highlighted in golden color. **(B)** Reduced *PMEPA1* gene expression correlates with *PMEPA1* gene methylation.

established methylated gene since its methylation status has been established for these CaP cell lines.^{23,29} The results indicated that *PMEPA1* is methylated only in AR positive cell lines and not in AR negatives (**Fig. 4A**). Consistent with previous reports,^{29,30} this study also showed that *GSTP1* is methylated in both AR positive and negative CaP cell lines. These observations suggest a cellular context dependent relationship between *PMEPA1* methylation and AR and therefore gain of AR function may be favored by decreased *PMEPA1* expression involving epigenetic mechanisms such as DNA methylation.

PMEPA1 expression is induced by the DNA methyltransferase inhibitor decitabine in AR positive CaP cell lines

PMEPA1 methylation may lead to the silencing of *PMEPA1* resulting in elevated AR protein levels and AR signaling. To further test this hypothesis, we treated the VCaP, LNCaP, and LAPC4 cell lines with low doses of decitabine. Western blot analysis revealed dose-dependent induction of *PMEPA1* protein in response to decitabine treatment (**Fig. 4B**). Consistent with the protein data qRT-PCR analysis also showed decitabine dose-dependent increases of *PMEPA1* mRNA expression (**Fig. 4B**). Taken together, these observations suggested that methylation contributes to the silencing of *PMEPA1* gene in AR positive CaP cell lines. We also monitored the response to decitabine treatment on AR protein levels. Consistent with the *PMEPA1*-AR negative feedback model decrease in the expression of AR protein was observed in response to decitabine treatment (**Fig. 4C**).

Silencing of *PMEPA1* may result in enhanced AR functions

PMEPA1 protein recruits AR to the NEDD4-1 ubiquitin ligase for degradation.¹⁶ Thus *PMEPA1* modulates levels of AR. Cancer-associated silencing of *PMEPA1* may result in elevated levels of AR and increased AR signaling (**Fig. 5A**). We have

Table 1. Clinicopathologic characteristics

Race	Caucasian American	African American
Number of patients	42	35
Gleason Sum		
6	17 (41.5)	14 (41.2)
7	15 (36.6)	16 (47.1)
8 to 10	9 (21.9)	4 (11.8)
Pathological T stage		
pT2	17 (42.5)	14 (41.2)
pT3–4	23 (57.5)	20 (58.8)
Surgical margin status		
Negative	39 (92.9)	28 (82.4)
Positive	3 (7.1)	6 (17.6)
PSA at diagnosis		
Median (range)	5.2 (1.1,23.4)	6.6 (3.2,98.7)
Seminal vesicle invasion		
Negative	39 (92.9)	28 (82.4)
Positive	3 (7.1)	6 (17.6)
Age at RP		
Mean (SD)	61.1 (8.4)	59.4 (8.3)
Median (range)	62.1 (40.2,73.6)	59.8 (45,71.9)
BMI		
Mean (SD)	26.2 (3.5)	27.3 (4.9)
Median (range)	26 (20,34)	28 (19,42)

evaluated the relationship of *PMEPA1* levels with AR activity. For monitoring the AR activity, we assessed the expression of the known AR regulated gene, *KLK3*(PSA) gene by *PMEPA1* knockdown in LNCaP cells. Reduced expression of *PMEPA1* protein was observed in *PMEPA1* siRNA treated LNCaP cells (Fig. 5B). As expected, both AR and PSA protein levels were increased in response to *PMEPA1* knockdown (Fig. 5B). Thus, the silencing of the *PMEPA1* gene leads to enhanced AR activity in CaP by eliminating a negative regulatory control of AR protein levels.

Discussion

Emerging data continues to underscore the critical roles of *PMEPA1* as an androgen regulated NEDD4 E3 ligase binding protein in maintaining AR protein levels in prostate epithelial cells.^{14–16,21} Therefore, decrease or loss of *PMEPA1* expression and function may have major impact on gain of AR function and CaP progression. The goal of this study was to elucidate the molecular basis for the reduced or lost expression of *PMEPA1* in CaP. The results presented here demonstrate that promoter methylation is a major mechanism involved in silencing the *PMEPA1* gene in CaP. The potential contribution of genomic methylation in the modulation of *PMEPA1* expression was further supported

using CaP cell lines. AR positive cells (LNCaP, LAPC4, and VCaP) showed methylation of the *PMEPA1* gene, suggesting that the methylation may indeed contribute to the repression of *PMEPA1*.²¹ Increased expression of *PMEPA1* in response to decitabine treatment of CaP cell lines was reflected both at mRNA and protein levels. The high frequency of methylation silencing of *PMEPA1* in CaP samples provides further support for the hypothesis that *PMEPA1* may negatively control prostate tumorigenesis through the AR axis (Fig. 5A and B). Similar to other cancers, methylation has been shown to play a major role in the CaP genome.³⁰ In fact, methylation and loss of expression of the *GSTP1*, a gene involved in the cellular redox maintenance, is present in a majority of CaPs. Moreover, *GSTP1* methylation represents one of the earliest genomic alterations in CaP onset.²⁵ Other genes of functional relevance have also been shown to be methylated in CaP.^{28–32}

Studies from our and other groups have shown that *PMEPA1* is a multifunctional protein.^{15–21} However, *PMEPA1* functions is likely cellular context dependent and may be different in the presence or absence of AR.²⁰ In normal prostate epithelial cells a negative feedback loop between AR and *PMEPA1*/NEDD4 regulates AR. In CaP, loss of *PMEPA1* appears to be critical in gain of AR function and CaP progression. Out of the AR context, a negative feedback loop between TGF- β and *PMEPA1* has been noted through direct interaction of *PMEPA1* with SMAD3/4,²⁰

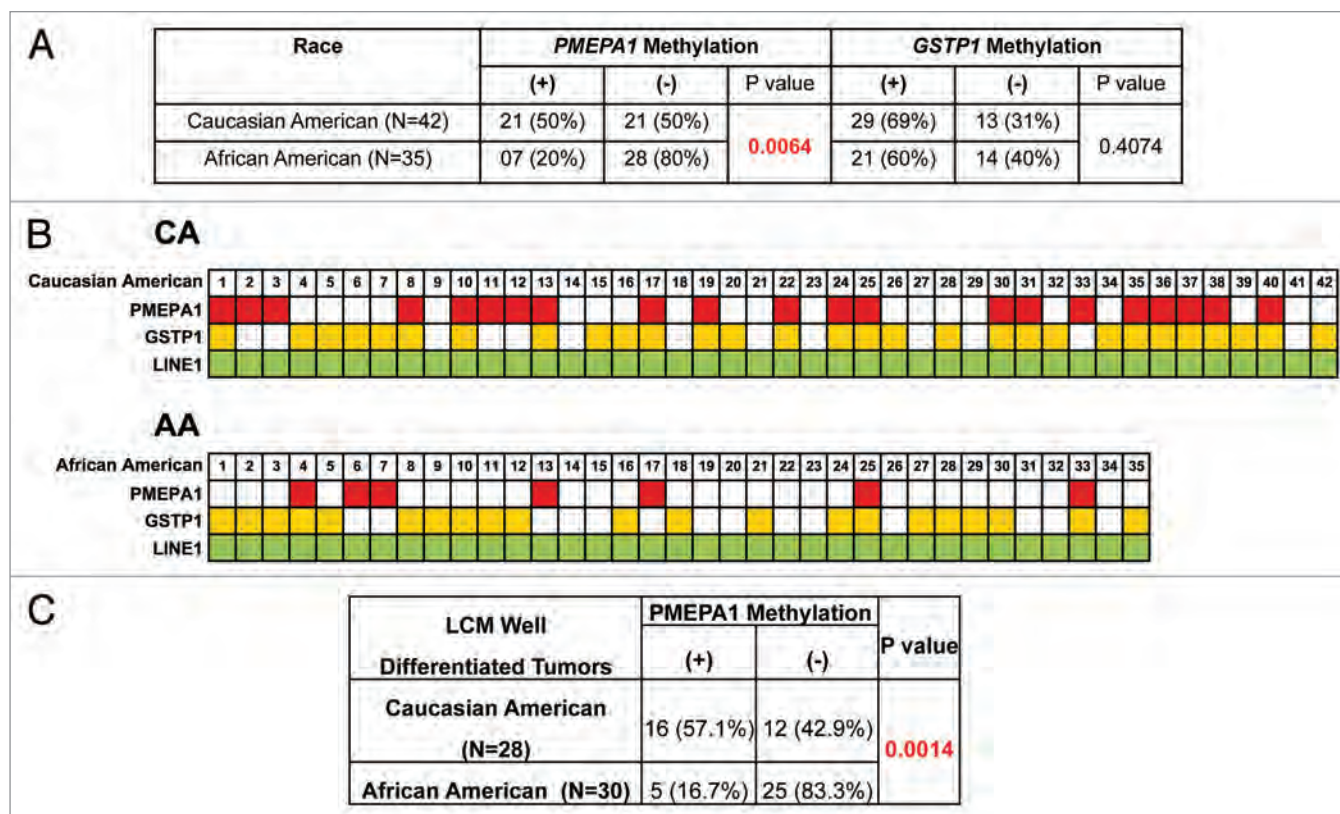


Figure 3. (A) Association of *PMEPA1* methylation with ethnicity. **(B)** Methylation status of *PMEPA1* and *GSTP1* gene in LCM selected prostate tumor DNA. The constitutively methylated *LINE1* repetitive element was used as quality control for input DNA. Methylated genes are marked with red (*PMEPA1*), yellow (*GSTP1*) and green (*LINE1*). **(C)** Correlation analyses of *PMEPA1* methylation status in CA and AA CaP patients with well differentiated tumors.

supporting cancer progression. Thus, gain or loss of *PMEPA1* expression and function may indeed cancer cell type dependent.

The differential methylation of *PMEPA1* gene between CA (50%) and AA patients (20%) was an unexpected finding. Emerging data on CaP genomes is beginning to clarify genomic differences between ethnic groups. CaP-associated somatogenetic and epigenetic differences between various ethnic groups are increasingly recognized.^{26,27,33-37} However, the distinct biology is not well understood and warrants further investigations, including the observed *PMEPA1* methylation difference between CA and AA patients.

A significant effort has been devoted toward developing inhibitors targeting epigenetic modification of the cancer genome including FDA approved DNMT inhibitors (azacitidine and decitabine) for Myelodysplastic Syndrome and HDAC inhibitors (vorinostat and romidepsin) for Cutaneous T Cell Lymphoma.^{38,39} With the increasing knowledge of epigenetic alterations linked to CaP, such as in *GSTP1* or AR axis, more targeted therapeutic strategies may be possible. *PMEPA1* may represent a new promising target for epigenetic drugs or new class of drugs complementing AR axis inhibitors.

In conclusion, the *PMEPA1* gene is methylated in CaP primary prostate tumors suggesting that methylation may contribute to the silencing of *PMEPA1*. *PMEPA1* gene expression levels correlated with its DNA methylation status. *PMEPA1* methylation status displayed an intriguing difference between CA and

AA patients. These data, along with our earlier observation, indicate that reduced or lost *PMEPA1* expression in CaP cells may lead to elevated AR levels. Our study provides insights into the role of methylation in the CaP-associated silencing of *PMEPA1* with potential impact on the AR axis in malignant prostate. Targeting AR for degradation by *PMEPA1* may synergize current therapeutic approaches.

Materials and Methods

Prostate cancer specimens and clinico-pathological data

Radical prostatectomy (RP) specimens and clinico-pathological data were obtained from patients enrolled in the Center for Prostate Disease Research (CPDR) from 1996 to 2010 under an institutional review board-approved protocol at the Walter Reed National Military Medical Center (WRNMMC) and Uniformed Services University of the Health Sciences (USUHS) (Table 1). Optimum cutting temperature (OCT) embedded RP tissue specimens from 77 patients, including 42 CA and 35 AA, were analyzed in this study. None of these patients had received prior androgen deprivation therapy. The biochemical recurrence was defined as two consecutive post-operative PSA values (≥ 0.2 ng/mL) measured at ≥ 8 wk post-operatively.

LCM derived normal and malignant prostate epithelial cells were obtained from OCT embedded frozen sections as described

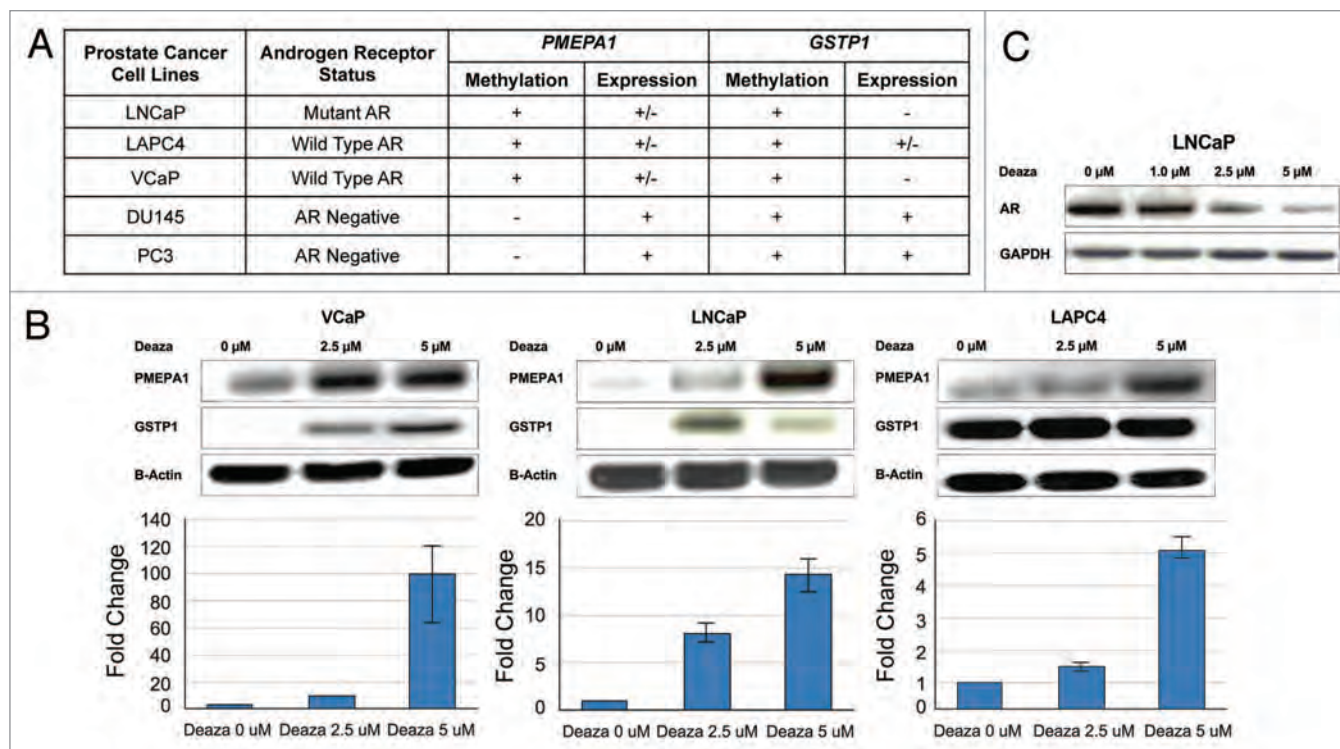


Figure 4. (A) Methylation (+ or -) and expression (+, - or +/- intermediate) status of *PMEPA1* and *GSTP1* genes in AR positive and AR negative prostate cancer cell lines. VCaP, LNCaP, and LAPC4 cells harbor low *PMEPA1* expression whereas no *GSTP1* was detected in VCaP and LNCaP cells. (B) *PMEPA1* expression is induced by the DNA methyl transferase inhibitor decitabine in VCaP, LNCaP, and LAPC4 cells after 14 d treatment. *PMEPA1*, *GSTP1*, and Beta-Actin protein levels were analyzed by immunoblot assays (upper panels). Gene expression was monitored by qRT-PCR (lower panels) and is shown as fold changes normalized to GAPDH control. (C) Reduced AR protein levels in LNCaP cells in response to decitabine treatment.

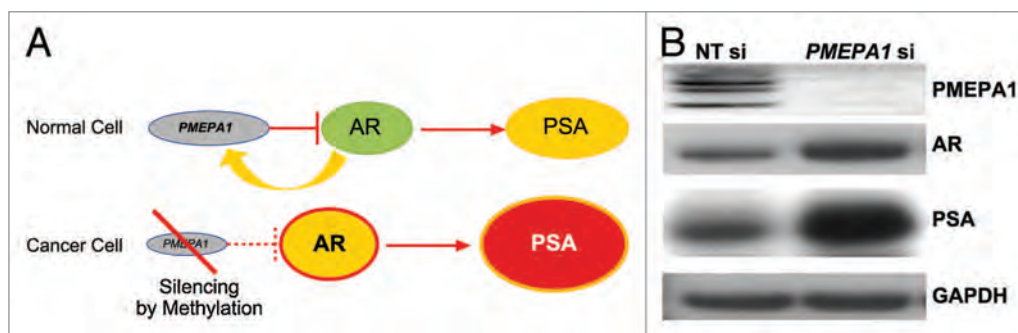


Figure 5. (A) Silencing of the *PMEPA1* gene disrupts a negative control over AR leading to enhanced AR activity resulting in elevated levels of PSA. (B) AR and PSA protein levels are increased in response to *PMEPA1* knockdown in LNCaP cells.

previously.⁴⁰⁻⁴³ Briefly, specimens obtained immediately after surgical resection of prostate, were OCT embedded and frozen on glass slides placed on dry ice. Benign and malignant cells were isolated by LCM using 6 micron frozen tissue sections archived in CPDR frozen tissue section slide library stored at -80 °C. The strategy for the evaluation of methylation and expression is described in Figure 6A and B.

Isolation of genomic DNA and total RNA from LCM derived prostate epithelial cells

Total RNA from the LCM derived specimens was isolated, purified and quantified.^{41,42} For genomic DNA, LCM-derived cells were lysed (buffer containing 10 mM Tris, 1 mM EDTA,

1% Tween 20) and digested with proteinase K (1 mg/ml; Roche Applied Science) at 37 °C overnight.⁴⁴ DNA was precipitated by adding 1/10th volume of 3M sodium acetate pH 5.2 (Sigma-Aldrich) and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) (Sigma-Aldrich). For optimal recovery the DNA was precipitated by adding 2 μg of glycogen (Roche Applied Science) and equal volume of chilled isopropanol (Sigma-Aldrich). After centrifugation the DNA pellets were washed with 70% ethanol (Sigma-Aldrich) and resuspended in 25 μl of TE buffer pH 8.0 (Sigma-Aldrich). DNA quantitation of all samples was performed by using Picogreen dye (Quant-iT™ PicoGreen® dsDNA Reagent and Kits, Life Technology) and

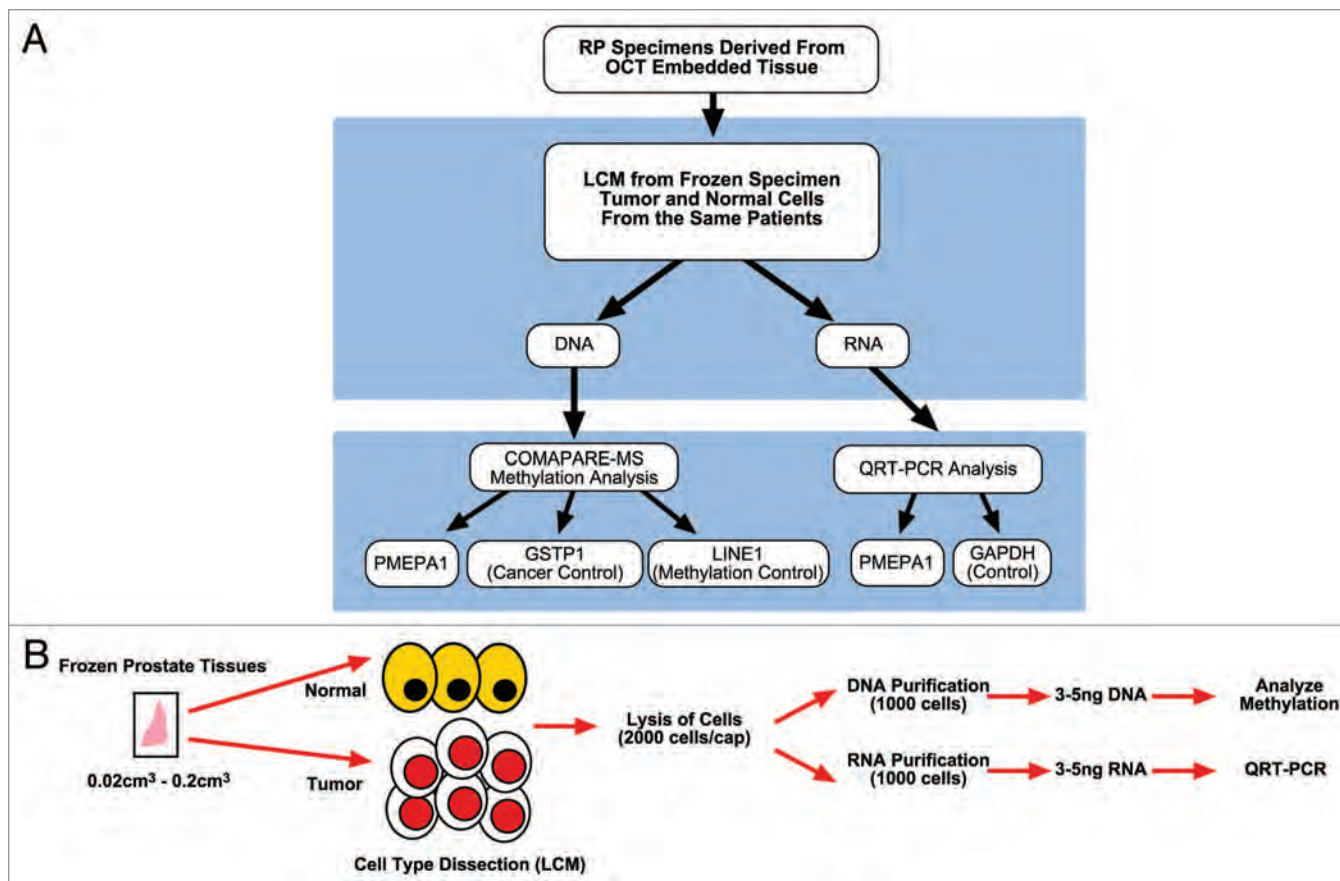


Figure 6. (A) Flowchart of DNA methylation and quantitative gene expression analysis in prostate tumor specimens. **(B)** LCM, determination of methylation by combination of methylated DNA precipitation and methylation-sensitive restriction cleavage (COMPARE-MS), and quantitative gene expression analyses.

confirmed with 260/280 ratio by NanoDrop 2000c spectrophotometer (Thermo Scientific).

DNA Methylation assay for genomic DNA isolated from LCM derived prostate cancer cells and prostate cancer cell lines

Based on publicly available genome wide methylation collected by the ENCODE consortium (<http://genome.ucsc.edu>) as well as by whole genome methylation analyses (Haffner MC and Yegansubramanian S., personal communication) the first intronic region of *PMEPA1* was selected for the analyses by the consistent differential methylation between LNCaP and primary prostate epithelium-derived PrEC cells and by the linear detection range in assaying low quantity of input DNA (Fig. S1A and B).

A combination of COMPARE-MS method, followed by Quantitative real time-Polymerase Chain Reaction (Q-PCR) was used for the evaluation of the DNA methylation.²³ Two ng of purified genomic DNA from LCM derived prostate tumor cells was digested with 10U each of AluI and HhaI (New England Biolabs) at 37 °C for 3 h followed by heat inactivation of enzymes at 80 °C for 20 min. Methylated genomic DNA fragments were captured by recombinant MBD2 protein derived methyl-binding domain polypeptides immobilized on magnetic beads. In parallel, 2 ng of genomic DNA from normal male white

blood cells (WBC), treated with 4U of CpG Methylase (M.SssI) enzyme (Zymo Research) and untreated DNA were also analyzed, serving as positive and negative controls, respectively.

Primers designed for the methylated CpG region of the *PMEPA1* gene (forward primer, 5'-CGTCTGCCCT GCTTAAACT; reverse primer, 5'-TTTGGGAGAT GGGTTTTCAC) and *GSTP1* (forward primer, 5'-GGGACCCTCC AGAAGAGC; reverse primer, 5'-ACTCACTGGT GGCGAAGACT) were used for SYBR Green-based Q-PCR. *LINE1* repetitive elements, which are highly methylated in human genomic DNA, were used as positive control to ensure the recovery of methylated DNA during the COMPARE-MS assay. *LINE1* promoter (GenBank accession X58075) was amplified by the following primer set: forward primer 5'-CGCAGAAGAC GGGTGATTTC-3' and reverse primer 5'-CCGTCACCCC TTTCTTTGAC-3'.

Q-PCR was performed in duplicate with 25 µL reactions containing 1X IQ Sybr-Green Supermix (Bio Rad) and 0.4 µM forward and reverse primers under the following cycling conditions: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s on Stratagene Mx3000P (Agilent Technologies Inc.). The COMPARE-MS assay was used to analyze *PMEPA1*, *GSTP1*, and *LINE1* methylation in LCM-derived prostate tumor cell and AR positive and AR negative CaP cell

lines, VCaP, LNCaP, LAPC4, and DU145, PC3, respectively. The methylation status was categorized binary as present or absent. The methylation levels were normalized to the signal generated by an equal input amount of the positive control.²³

Quantitative real-time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) analysis

TaqMan qRT-PCR using RNA from LCM derived tumor and normal epithelial cells was performed as described previously.⁴⁰⁻⁴³ The expression of *GAPDH* as the RNA input control and the target gene expression were simultaneously analyzed for each sample (in duplicates). A negative control without reverse-transcriptase enzyme was included for each specimen to rule out background signal from potential genomic DNA contamination.

qRT-PCR analysis of *PMEPA1* and *GAPDH* expression was performed on Stratagene Mx3000P (Agilent Technologies, Inc.). The PCR primers for *PMEPA1* were 5'-CATGATCCCC GAGCTGCT-3' (forward) and 5'-TGATCTGAAC AAAGTCCAGC TCC-3', (reverse) and the FAM-labeled probe was 5'-AGGCGGACAG TCTCCTGCGA AA-3'. *GAPDH* primers and probe mix were obtained from Life Technologies. All qRT-PCR reactions were performed in triplicate and data were analyzed by using MxPro v.3.2 software (Agilent Technologies Inc.). Amplification plots were evaluated and threshold cycle (CT) was set for each experiment. Multiplex measurements for target gene and *GAPDH* were averaged across triplicates and used to calculate standard deviation for each set. Subtraction of averaged *GAPDH* CT from averaged target gene CT yielded the Δ CT. Expression fold-change differences between normal and tumor was calculated by comparing Δ CT values among matched sample sets.

Cell culture

Human prostate tumor cell lines, VCaP, LNCaP were purchased from American Type Culture Collection (ATCC) and LAPC4 kindly provided by Dr Charles L. Sawyers (UCLA, CA). VCaP and LNCaP cells were maintained in DMEM (ATCC), and LAPC4 cells in RPMI-1640 (ATCC) supplemented with 10% of fetal bovine serum (Invitrogen) in a humidified CO₂ (5%) incubator at 37 °C. Cell culture and cell line treatment studies have been performed in two independent sets of experiments.

Decitabine (5-aza-2×-deoxycytidine) treatment

One million cells were seeded in T75 flasks in triplicate in the cell growth medium containing 0 μ M, 2.5 μ M and 5 μ M of the DNA methyl transferase inhibitor, decitabine (5-aza-2×-deoxycytidine; Sigma-Aldrich) for 14 d. The media with decitabine were changed every 48 h. After 14 d cells were harvested for isolation of genomic DNA, total RNA and protein.

Transfection and siRNA knock-down of PMEPA1

The LNCaP cells were seeded into 10 cm culture dishes (FALCON, Becton Dickinson) at the density of 1.5×10^6 cells/ dish. The cells were incubated at 37 °C, 5% CO₂ for 48 h. After incubation the cells were transfected with 50 nM of control Non-targeting (NT) siRNA and PMEPA1si RNA (5'-GTTATCACCA CGTTATATA-3') (Dharmacon) by using Lipofectamine 2000 (Life Technologies). The cells were incubated with transfection complex at 37 °C, 5% CO₂ for 12 h.

After incubation the transfection complex was removed and the cells were replenished with fresh complete medium. The cells were harvested 48 h post-transfection for western blot assay.

Western blot analysis

Cells from the cell culture experiments were lysed in Mammalian Protein Extraction Reagent (M-PER) (Pierce from Thermo Fisher Scientific Inc.) in the presence of protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Cell lysates equivalent to 25 μ g of total protein were separated on 4–12% Bis-Tris gel (Life Technologies) and transferred to PVDF membrane. Membranes were incubated with primary antibodies: anti-PMEPA1 monoclonal antibody (Novus Biologicals), anti-GSTP1 polyclonal antibody (US Biologicals), anti- β -Actin monoclonal antibody (Cell Signaling), anti-PSA polyclonal antibody (Dako) and anti-AR polyclonal antibody (Santa Cruz Biotech) at 4 °C overnight and were washed before being treated with respective secondary antibodies (GE Healthcare Biosciences). Western blots were visualized by the Amersham ECL western blot detection reagent (GE Healthcare Biosciences).⁴⁵

Statistical analysis

Seventy-seven CaP patients (42 CA and 35 AA) who underwent RP for primary treatment were analyzed for *PMEPA1* and *GSTP1* methylation status. The cohort was designed based on our initial observations indicating higher frequency of *PMEPA1* methylation in CA patients (42 CA and 8 AA). This minimum sample size ($n = 77$) was determined by statistical power calculation (90% power with 0.05 α , two sided chi square test). RNA and DNA were analyzed from the same specimens. *PMEPA1* mRNA expression was normalized to *GAPDH* and reported as fold change between matched normal and tumor pairs. *PMEPA1* and *GSTP1* genomic DNA methylation data were reported as present or absent. Distributions of other clinico-pathological variables were examined by using Chi-square or Fisher exact tests.

Chi-square test was used to evaluate the association of *PMEPA1* and *GSTP1* methylation and *PMEPA1* expression with race/ethnicity. Chi-square test was also used to examine the association of *PMEPA1* methylation with *PMEPA1* expression. Association of the combination of *PMEPA1* methylation and expression groups (methylation-/high expression, methylation+/low expression) with race was tested by using Fisher's exact test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/28710

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